



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 9/72, G01N 33/68, 33/566, A61K 38/49, G01N 33/50</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/01802</b> <b>(43) International Publication Date:</b> 13 January 2000 (13.01.00)
<b>(21) International Application Number:</b> PCT/DK99/00377 <b>(22) International Filing Date:</b> 1 July 1999 (01.07.99) <b>(30) Priority Data:</b> PA 1998 00874 1 July 1998 (01.07.98) <b>DK</b> <b>(71) Applicant (for all designated States except US):</b> CANCERFORSKNINGSFONDEN AF 1989 (FONDEN TIL FREMME AF EKSPERIMENTEL CANCERFORSKNING) [DK/DK]; Michael Rostock, Vester Voldgade 90, DK-1552 Copenhagen V (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> PLOUG, Michael [DK/DK]; Nørrebrogade 63, 2. th., DK-2200 Copenhagen N (DK). ØSTERGAARD, Søren [DK/DK]; Borups Allé 235D, st.tv., DK-2400 Copenhagen NV (DK). HOLM, Arne [DK/DK]; Margrethevej 19, DK-2840 Holte (DK). HOLST-HANSEN, Claus [DK/DK]; Hostrupvej 5, 1.tv., DK-1950 Frederiksberg C (DK). STEPHENS, Ross, W. [DK/DK]; Broholms Allé 2. th., DK-2920 Charlottenlund (DK). DANØ, Keld [DK/DK]; L. E. Bruuhnsvej 20, DK-2920 Charlottenlund (DK).		<b>(74) Agent:</b> PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annæ Plads 11, P.O. Box 3007, DK-1021 Copenhagen K (DK). <b>(81) Designated States:</b> AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> <b>(88) Date of publication of the international search report:</b> 27 April 2000 (27.04.00)
<b>(54) Title:</b> PEPTIDE ANTAGONISTS OF THE HUMAN UROKINASE RECEPTOR AND METHOD FOR SELECTING THEM		
<b>(57) Abstract</b> <p>A novel set of inhibitors of the binding interaction between human urokinase plasminogen activator (uPA) and its cell surface receptor (uPAR) has been developed. The inhibitors comprise of peptide fragments, monomeric or in multiple copies attached to a common scaffold, in which the amino acid sequence may include uncommon substituted amino acids to partially comprise of peptoid sequences. The present invention also relates to the use of such peptides in therapy, in particular for the treatment of cancer, having developed a modified non-human mammalian receptor to which the novel inhibitors are antagonistic. The invention also relates to a method for selecting peptides antagonizing the binding between uPA and uPAR.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

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CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>20411 PC 1</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/DK 99/ 00377</b>	International filing date (day/month/year) <b>01/07/1999</b>	(Earliest) Priority Date (day/month/year) <b>01/07/1998</b>
Applicant <b>CANCERFORSKNINGSFONDEN AF 1989 (FONDEN TIL FREMME;</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 57 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

### 1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☒ furnished subsequently to this Authority in written form.

☒ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (see Box II).

### 4. With regard to the title,

☐ the text is approved as submitted by the applicant.

☒ the text has been established by this Authority to read as follows:

**PEPTIDE ANTAGONISTS OF THE HUMAN UROKINASE RECEPTOR AND METHOD FOR SELECTING THEM**

### 5. With regard to the abstract,

☐ the text is approved as submitted by the applicant.

☒ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

### 6. The figure of the drawings to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 99/00377

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 7 C12N9/72 A61K38/49 G01N33/68 G01N33/50 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 05257 A (CHIRON CORP ; ROSENBERG STEVEN (US); SPEAR KERRY L (US); VALERIO RO) 13 February 1997 cited in the application see the whole document	1-40
A	GOODSON R J ET AL: "HIGH-AFFINITY UROKINASE RECEPTOR ANTAGONISTS IDENTIFIED WITH BACTERIOPHAGE PEPTIDE DISPLAY" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 91, no. 15, page 7129-7133 XP000574761 cited in the application see the whole document	1-40
-/-		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

**\* Special categories of cited documents :**

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

13 December 1999

Date of mailing of the international search report

07.03.2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

GROENENDIJK, M



# INTERNATIONAL SEARCH REPORT

Intern    nal Application No  
PCT/DK 99/00377

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 35969 A (CHIRON CORP ;BRIGHAM & WOMENS HOSPITAL (US); ROSENBERG STEVEN (US)) 2 October 1997 cited in the application see the whole document ---	1-40
A	PLOUG E.A.: "Photoaffinity labelling of the human receptor for urokinase-type plasminogen activator using a decapeptide antagonist. Evidence for a composite ligand-binding site and a short interdomain separation" BIOCHEMISTRY, vol. 37, no. 11, 17 March 1998, EASTON, PA US, pages 3612-3622, XP002124882 see the whole document ---	1-40
A	FAUCHÈRE E.A.: "Amino acid sidechain parameters for correlation studies in biology and pharmacology" INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH, vol. 32, no. 4, October 1988, COPENHAGEN DK, pages 269-278, XP000857464 cited in the application see the whole document -----	13-16

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00377

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-40

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/ DK 99/00377

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box 3.

Claims Nos.: 1-24,26-35,37-40(all partially)

Present claims 1-24,26-35 and 37 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. In fact, the claims also contain so many options, variables and possible permutations, that also a lack of clarity and/or conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), disclosed and supported, namely the compounds defined in the examples and closely related homologous compounds (Art.17(2)(b) PCT).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/DK 99/00377

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-40

peptides inhibiting the binding between uPA and uPAR, their compositions and use

2. Claims: 41-58

Method for selecting peptides inhibiting the binding between uPA and uPAR as defined in the claims 41-58

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 99/00377

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9705257 A	13-02-1997	AU 6505996 A EP 0842281 A	26-02-1997 20-05-1998
WO 9735969 A	02-10-1997	AU 2597897 A EP 0906419 A	17-10-1997 07-04-1999

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
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in its capacity as elected Office

Date of mailing (day/month/year) 29 February 2000 (29.02.00)	
International application No. PCT/DK99/00377	Applicant's or agent's file reference 20411 PC 1
International filing date (day/month/year) 01 July 1999 (01.07.99)	Priority date (day/month/year) 01 July 1998 (01.07.98)
Applicant PLOUG, Michael et al	

1. The designated Office is hereby notified of its election made:



in the demand filed with the International Preliminary Examining Authority on:

25 January 2000 (25.01.00)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was



was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer F. Baechler Telephone No.: (41-22) 338.83.38
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US cards

(courtesy copy of FBI sub. pg's)

OSB	Grach	✓COHN	1	Nat, 1 <sup>st</sup> pg of pub, drg(5), IPER, ISR, \$	1-2-01	AF
ilm	Bonaccorsi	✓BUER	1	Nat, dec, drg(2), ISR, IPE, &	1-2-01	OF
ilm	Ploug	✓PROU	1	Nat, drg(10) ISR, IPE, &	1-2-01	OF
ilm	Ueno	✓AOYB	8	Nat, p.m.N, s.m.N, drg(5), ISR, DEC, &	1-2-01	OF
OyB	Ebihara 2	✓YUAS	09/453,058	Drgs(5)	1-2-01	OF
ilm	Kalodner 1	✓COHN	09/167,547	IFF	1-2-01	OF
ilm	Wald 1	✓COHN	09/194,078	IFF	1-2-01	OF
ilm	Wald 1	✓COHN	09/194,078	Drg(1)	1-2-01	OF
(	D Amato 1B	SOCH	09/237,711		1-2-01	
OyB	Kisters 1	✓ISLE	09/101,817	CPA (2pgs)	1-2-01	OF
OyB	Murata 5	✓SIXS	09/284,394	CPA, Amendment,	1-2-01	OF
OSB	Nathan	✓COHN	8	Rev. APP, \$, Drg(5), spec	1-2-01	OF
ilm	Antlo 1	✓BRAN	09/258,390	Amend A.F.	1-2-01	OF
ilm	Chen 287	✓DIRE	09/706,821	Pri. Doc. (1)	1-2-01	OF
ilm	Hausman 2	✓COHN	09/698,122	Pri. Doc. (1)	1-2-01	OF
ilm	Hausman 2	✓COHN	09/698,122	Pri. Doc. (1)	1-2-01	OF
ilm	Akahwi 1	✓	09/705,712	Pri. Doc. (1)	1-2-01	OF
ilm	Thome 1	✓LAUL	09/642,823	Pri. Doc. (1)	1-2-01	OF
ilm	Cheng 44	✓DIRE	09/702,661	Pri. Doc (1)	1-2-01	OF
ilm	Chen 285	✓DIRE	09/662,877	Pri. Doc. (1)	1-2-01	OF
ilm	Thong 3	✓RAU	09/604,294	Pri. Doc (1)	1-2-01	OF
ilm	Ushio 2	✓SUMA	09/716,356	Rep. Priority	1-2-01	OF
ilm	Mazor 3	✓YEDA	08/068,379	Status Req.	1-2-01	OF
ilm	Fiordelisi 1	✓BUER	09/194,026	2nd Status Req.	1-2-01	OF
ilm	Medan 3	✓COHN	09/329,799	Status Request	1-2-01	OF
ilm	Cohen 38	✓COHN	09/324,101	Status Request	1-2-01	OF
ilm	Novick 7B	✓INL2	08/472,402	Status Request	1-2-01	OF
ilm	Blumental 1	✓COHN	09/328,392	Status Req	1-2-01	OF
ilm	Spindler 1	✓KIRT	09/320,704	Status Req	1-2-01	OF
ilm	Halo 1	✓Nede	09/600,449	Log	1-2-01	OF
ilm	Thome 1	✓LAUL	09/642,823	Log	1-2-01	OF
ilm	Livneh 1A	✓YEDA	09/627,399	Log	1-2-01	OF
ilm	Fukusawa 1	✓KANF	09/651,029	Log	1-2-01	OF

APPENDIX A

ilm	Graupner 2	✓RAU	09/599,257	Log	1-2-01	OB
ilm	Garcia - Martin 1	✓OCHA	09/623,857	Log	1-2-01	OB
ilm	Watanabe 26	✓YUAS	09/647,874	Log	1-2-01	OB
ilm	Fumamelli 5	✓LUNA	09/618,625	Log	1-2-01	OB
ilm	Laurila 2	✓OYBO	09/623,380	Log	1-2-01	OB
ilm	Ferretti 1	✓SACL	09/554,248	Log	1-2-01	OB
ilm	Keydel 2	✓KRB	09/639,329	Log	1-2-01	OB
ilm	Azagury 4	✓CONN	09/327,379	Status Req.	1-2-01	OB
ilm	Eis-Schwartz 14A	✓EDA	09/643,909	Log	1-2-01	OB
ilm	Nirahara 1	✓KANE	09/668,296	Log	1-2-01	OB
ilm	Tabacco 2	✓ECHO	09/641,240	Log	1-2-01	OB
ilm	Margalit 1	✓CONN	60/230,457	Log	1-2-01	OB
ilm	Kazohara 3	✓SATO	09/670,576	Log	1-2-01	OB
ilm	Sumiya 1A	✓ASAM	5,645,804	1st M. Fee	1-2-01	OB
ilm	Richard 3	✓	5,225,041	2nd M. Fee	1-2-01	OB
ilm	Amirav 1	✓	4,845,367	3rd M. Fee	1-2-01	OB
ilm	El Tayar 4	✓LAVO	09/319,014	Restriction Resp	1-2-01	OB
ilm	Muzio 1	✓SERI	09/297,369	Chg. Address	1-2-01	OB
ilm	Depyro 1	✓MIRO	75/611,004	Ext. of Home (6 mo.)	1-2-01	OB
ilm	Contraire 1	✓	75/215,682	Req. deletion of 1b/Req. w/D of NOA	1-2-01	OB
ilm	Charvet 1	✓GM	75/491,861	Req. to formulate App	1-2-01	OB
ilm	NNT 1	✓ECI	76/054,422	Req. for OFL	1-2-01	OB
ilm	Miyouchi 3	✓YUAS	09/555,815	Req. Corr. Log	1-2-01	OB
ilm	Feng	✓DIRE	9	App. drg (4), SML, DEC, B	1-2-01	OB
ilm	Akita	✓SUMA	1C	Div App, drg (1), Seq. SLD, PMN, IDS, DEC#	1-2-01	OB
ilm	Okayama 2	✓ONTA	09/646,349	Mag facts w/dec + 1 ext	1-2-01	OB
OB	Torok 13a	✓BRAN		Spec, drg, pres. and, DL,	1-2-01	OB
OB	Ebihara - 2	✓YUAS	09/453,058	Amendment (ext), B	1-2-01	OB
OB	Huesch	✓	09/242,340	IDS	1-2-01	OB



# OLIVIA J. BANE

## OBJECTIVE

Obtain a position that will utilize my organizational skills and provide an opportunity for career advancement. To unite with a company that requires a strong, knowledgeable individual and where I will be valued as a team player. To work for a company that desires someone who has excellent communication and interpersonal skills, while maintaining a positive attitude.

## EDUCATION

1995 - 1999 University of California at Davis Davis, CA  
*Bachelor of Arts in Psychology*

- Minor in Sociology
- 3.2 GPA

## AWARDS RECEIVED

Scholarship recipient of the Dumbarton and Ohlone Chapters of ABWA (American Business Woman of America).

## WORK EXPERIENCE

1998 - 1999 University of California at Davis Sacramento, Ca  
*Research Assistant and Coordinator*

- Performed field research for a Domestic Violence Study at the Rio Cosummes Correctional Center with an emphasis on conducting clinical interviews and administering personality assessment questionnaires.
- Executed programs involving data entry, analysis and the scoring of results.

1999 Imtrek Sacramento, Ca  
*Temporary Administrative Assistant*

- Used Alchemy software to edit and store multiple company's asset's

1997 - 1999 Café Roma Davis, Ca  
*Shift Leader / Assistant Manager*

- In charge of training and weekly scheduling.
- Responsible for handling inventory and ordering products needed.

1996 CALPIRG

Santa Cruz, Ca

Pacific Research Organizing and Development Division

*Field Petitioner*

- Worked as an initiative organizer and field petitioner.

#### SUMMARY OF SKILLS AND STRENGTHS

Working knowledge of the following computer programs:

- Microsoft Office to include Outlook
- Word Perfect
- Windows 95/98

Enthusiastic, energetic, and willing to assume increased responsibilities.

Able to diagnose problems quickly and provide appropriate and efficient solutions.

References available upon request.



There was a 30 min  
Train Delay on the  
yellow line

Jeffrey Johnson  
Officer, Badge No. 236 (703) 260-4111  
Field Operations Bureau  
Metro Transit Police Department

Washington Metropolitan Area Transit Authority  
600 Fifth Street, NW  
Washington, DC 20001



#### Incident Report Information

Dispatch 962-2121: 24 hours, 7 days/week

Records 962-2125: 8 a.m.-4:00 p.m., Mon.-Fri.

Reports available 5 working days after incident.

Incident No. \_\_\_\_\_  
Date 01-02-01  
Time 1145PM  
Location \_\_\_\_\_  
Officer J. J. Johnson

# The RideGuide



TEXT ONLY VERSION

It's easy as

**1 2 3**

**3**

Here's your best route...

From: **DUPONT CIRCLE  
METRO**To: **METRO CENTER  
METRO**

## ITINERARY - #1

Take RED LINE Towards GLENMONT  
METRO

<b>Depart:</b>	DUPONT CIRCLE METRO STATION	At 11:00 PM
<b>Arrive:</b>	METRO CENTER METRO STATION	At 11:04 PM

### REGULAR FARE

Bus Fare \$ 0.00  
Rail Fare \$ 1.10  
Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**

### SENIOR / DISABLED FARE

Bus Fare \$ 0.00  
Rail Fare \$ 0.55  
Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**



Need help?



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Start Over

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## ITINERARY - #2

Take 42 Metro Bus Towards METRO  
CENTER

<b>Depart:</b>	DUPONT CIRCLE METRO STATION	At 11:11 PM
<b>Arrive:</b>	NW H ST & NW 13TH ST	At 11:21 PM



**Walk** Walk 0.1 mile S to  
METRO CENTER METRO

[DETAILS](#)

### REGULAR FARE

Bus Fare \$ 1.10  
Rail Fare \$ 0.00  
Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**

### SENIOR / DISABLED FARE

Bus Fare \$ 0.50  
Rail Fare \$ 0.00  
Transfer Fee \$ 0.00  
**TOTAL: \$ 0.50**

APPENDIX D

# The RideGuide



TEXT ONLY VERSION

It's easy as

**1 2 3**

**3**
**Here's your best route...**

 From: **METRO CENTER  
METRO**

 To: **L'ENFANT PLAZA  
METRO**


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**ITINERARY - #1**

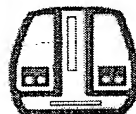
 Take BLUE LINE Towards ADDISON  
-ROAD


<b>Depart:</b>	METRO CENTER METRO STATION	At 11:07 PM
<b>Arrive:</b>	L'ENFANT PLAZA METRO STATION	At 11:11 PM

**REGULAR FARE**

 Bus Fare \$ 0.00  
 Rail Fare \$ 1.10  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**
**SENIOR/DISABLED FARE**

 Bus Fare \$ 0.00  
 Rail Fare \$ 0.55  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**
[BACK TO TOP](#)
**ITINERARY - #2**

 Take ORANGE LINE Towards NEW  
CARROLLTON


<b>Depart:</b>	METRO CENTER METRO STATION	At 11:17 PM
<b>Arrive:</b>	L'ENFANT PLAZA METRO STATION	At 11:22 PM

**REGULAR FARE**

 Bus Fare \$ 0.00  
 Rail Fare \$ 1.10  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**
**SENIOR/DISABLED FARE**

 Bus Fare \$ 0.00  
 Rail Fare \$ 0.55  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**
[BACK TO TOP](#)

APPENDIX E

# The RideGuide



TEXT ONLY VERSION

It's easy as

**1 2 3**


# 3

**Here's your best route...**
**From: L'ENFANT PLAZA  
METRO**
**To: CRYSTAL CITY  
METRO**

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**Start Over**

## ITINERARY - #1

**Take BLUE LINE Towards FRANCONIA**

**Depart:** L'ENFANT PLAZA METRO STATION At 11:27 PM

**Arrive:** CRYSTAL CITY METRO STATION At 11:47 PM

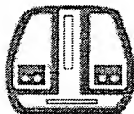
### REGULAR FARE

 Bus Fare \$ 0.00  
 Rail Fare \$ 1.10  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**

### SENIOR / DISABLED FARE

 Bus Fare \$ 0.00  
 Rail Fare \$ 0.55  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**
[BACK TO TOP](#)

## ITINERARY - #2

**Take YELLOW LINE Towards  
HUNTINGTON**

**Depart:** L'ENFANT PLAZA METRO STATION At 11:27 PM

**Arrive:** CRYSTAL CITY METRO STATION At 11:36 PM

### REGULAR FARE

 Bus Fare \$ 0.00  
 Rail Fare \$ 1.10  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**

### SENIOR / DISABLED FARE

 Bus Fare \$ 0.00  
 Rail Fare \$ 0.55  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**
[BACK TO TOP](#)

APPENDIX F

# The RideGuide



TEXT ONLY VERSION

It's easy as

**1 2 3**

**3**
**Here's your best route...**

 From: **DUPONT CIRCLE  
METRO**

 To: **METRO CENTER  
METRO**


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Start Over

## ITINERARY - #1

 Take RED LINE Towards GLENMONT  
METRO

**Depart:** DUPONT CIRCLE  
METRO STATION At 11:15  
PM

**Arrive:** METRO CENTER  
METRO STATION At 11:19  
PM

### REGULAR FARE

 Bus Fare \$ 0.00  
 Rail Fare \$ 1.10  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**

### SENIOR / DISABLED FARE

 Bus Fare \$ 0.00  
 Rail Fare \$ 0.55  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**
[BACK TO TOP](#)

## ITINERARY - #2

 Take 42 Metro Bus Towards METRO  
CENTER

**Depart:** DUPONT CIRCLE  
METRO STATION At 11:11  
PM

**Arrive:** NW H ST & NW  
13TH ST At 11:21  
PM

**Walk** Walk 0.1 mile S to  
METRO CENTER METRO
[DETAILS](#)

### REGULAR FARE

 Bus Fare \$ 1.10  
 Rail Fare \$ 0.00  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**

### SENIOR / DISABLED FARE

 Bus Fare \$ 0.50  
 Rail Fare \$ 0.00  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.50**

APPENDIX G

# The RideGuide



TEXT ONLY VERSION

It's easy as

**1 2 3**

**3**

Here's your best route...

From: METRO CENTER  
METROTo: L'ENFANT PLAZA  
METRO

## ITINERARY - #1

Take BLUE LINE Towards ADDISON  
ROAD

<b>Depart:</b>	METRO CENTER METRO STATION	At 11:26 PM
<b>Arrive:</b>	L'ENFANT PLAZA METRO STATION	At 11:30 PM

### REGULAR FARE

Bus Fare \$ 0.00  
 Rail Fare \$ 1.10  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**

### SENIOR / DISABLED FARE

Bus Fare \$ 0.00  
 Rail Fare \$ 0.55  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**



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APPENDIX H

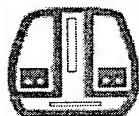


**ITINERARY - #2**

Take BLUE LINE Towards FRANCONIA

**Depart:** L'ENFANT PLAZA METRO STATION At 11:48 PM**Arrive:** CRYSTAL CITY METRO STATION At 12:08 AM**REGULAR FARE**Bus Fare \$ 0.00  
Rail Fare \$ 1.10  
Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10****SENIOR / DISABLED FARE**Bus Fare \$ 0.00  
Rail Fare \$ 0.55  
Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**[BACK TO TOP](#)**ITINERARY - #3**

Take YELLOW LINE Towards HUNTINGTON

**Depart:** L'ENFANT PLAZA METRO STATION At 11:59 PM**Arrive:** CRYSTAL CITY METRO STATION At 12:08 AM**REGULAR FARE**Bus Fare \$ 0.00  
Rail Fare \$ 1.10  
Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10****SENIOR / DISABLED FARE**Bus Fare \$ 0.00  
Rail Fare \$ 0.55  
Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**[BACK TO TOP](#)[Timetables](#) | [Maps](#) | [Fares](#) | [Talk to Metro](#) | [Metro Home Page](#) | [Feedback](#)

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APPENDIX I

# The Ride Guide



TEXT ONLY VERSION

It's easy as

**1 2 3**

**3**
**Here's your best route...**

 From: **DUPONT CIRCLE METRO** To: **GALLERY PLACE METRO**


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Start Over

**ITINERARY - #1**

Take RED LINE Towards GLENMONT METRO



<b>Depart:</b>	DUPONT CIRCLE METRO STATION	At 11:15 PM
<b>Arrive:</b>	GALLERY PLACE METRO STATION	At 11:21 PM

**REGULAR FARE**

 Bus Fare \$ 0.00  
 Rail Fare \$ 1.10  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**
**SENIOR / DISABLED FARE**

 Bus Fare \$ 0.00  
 Rail Fare \$ 0.55  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.55**
[BACK TO TOP](#)
**ITINERARY - #2**

Take 42 Metro Bus Towards METRO CENTER



<b>Depart:</b>	DUPONT CIRCLE METRO STATION	At 11:11 PM
<b>Arrive:</b>	NW 9TH ST & NW G ST	At 11:24 PM


**Walk** Walk 0.1 mile E to  
**GALLERY PLACE METRO**
[DETAILS](#)
**REGULAR FARE**

 Bus Fare \$ 1.10  
 Rail Fare \$ 0.00  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**
**SENIOR / DISABLED FARE**

 Bus Fare \$ 0.50  
 Rail Fare \$ 0.00  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.50**

APPENDIX J

1/9/01 12:08 P

# The RideGuide



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It's easy as

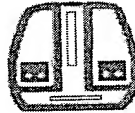
**1 2 3**

**3**

Here's your best route...

From: GALLERY PLACE  
METROTo: CRYSTAL CITY  
METRO

## ITINERARY - #1

Take YELLOW LINE Towards  
HUNTINGTON

<b>Depart:</b>	GALLERY PLACE METRO STATION	At 11:25 PM
<b>Arrive:</b>	CRYSTAL CITY METRO STATION	At 11:36 PM

### REGULAR FARE

Bus Fare \$ 0.00  
 Rail Fare \$ 1.10  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 1.10**

### SENIOR / DISABLED FARE

Bus Fare \$ 0.00  
 Rail Fare \$ 0.60  
 Transfer Fee \$ 0.00  
**TOTAL: \$ 0.60**



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APPENDIX K

**marianne Heins**

DATE 1402900000 TIME  
01/02/01 000000 22:22

BRICKSKELLER  
1623 22ND STREET NW  
WASHINGTON DC 20037  
(202) 293-1885

TRAN #  
575

AUTH  
06450

PRE-AUTH

ACCT. NUMBER

EXP

SVR. CHG TYPE

FOOD/BEV \$

56.27

TIP

TOTAL \$

SIGNATURE

OLIVIA JANE

THANK YOU

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BOTTOM COPY-CUSTOMER



- Each passenger must have a card.
- Use the same card for both entry and exit.
- Low farecard value may be increased at designated fare vendors in participating Rail Stations.
- This card can be returned and replaced with a regular farecard, but cannot be exchanged for cash.
- This card must remain in the possession of the passenger and be produced on demand by WMATA Employee or Police.

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WMATA  
600 Fifth Street, N.W.  
Washington D.C. 20001

900428762 C26J908

23 OCT. 2000

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference 20411 PC 1	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) <b>FOR FURTHER ACTION</b>	
International application No. PCT/DK99/00377	International filing date (day/month/year) 01/07/1999	Priority date (day/month/year) 01/07/1998
International Patent Classification (IPC) or national classification and IPC C12N9/72		
Applicant CANCERFORSKNINGSFONDEN AF 1989 ... et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.  
  
☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  25/01/2000	Date of completion of this report  19.10.2000
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Strobel, A  Telephone No. +49 89 2399 7362  

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/DK99/00377

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-52 as originally filed

**Claims, No.:**

1-58 as originally filed

**Drawings, sheets:**

1/10-10/10 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.  
☒ claims Nos. 1-24, 26-35, 37-40 (all partially); 41-58 (completely).

because:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/DK99/00377

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☒ no international search report has been established for the said claims Nos. 1-24, 26-35, 37-40 (all partially); 41-58 (completely).

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims	1-40
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-40
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-39
	No:	Claims	

**2. Citations and explanations**

**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

---

International application No. PCT/DK99/00377

**Re Item III**

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Present claims 1-24, 26-35, and 37-40 relate to an extremely large number of possible compounds, as already stated in the International Search Report. Said claims are not supported by the description over their whole width (Article 6 PCT). The International Search Report has been limited to the compounds disclosed in the description.
2. The applicant did not pay the required additional search fees for the search of claims 41-58. Therefore, the International Search Report is limited to claims 1-40.
3. Claims or parts of claims for which no International Search Report has been established need not be the subject of an International Preliminary Examination (Rule 66.1(e)PCT). In consequence, the Preliminary Examination covers only the proportion of the claimed compounds that has been disclosed in the examples of the description.

**Re Item V**

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

- D1: WO 97 05257 A (CHIRON CORP ; ROSENBERG STEVEN (US); SPEAR KERRY L (US); VALERIO RO) 13 February 1997 cited in the application
- D2: GOODSON R J ET AL: "HIGH-AFFINITY UROKINASE RECEPTOR ANTAGONISTS IDENTIFIED WITH BACTERIOPHAGE PEPTIDE DISPLAY" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 91, no. 15, page 7129-7133 XP000574761 cited in the application
- D3: PLOUG E.A.: "Photoaffinity labelling of the human receptor for urokinase-type plasminogen activator using a decapeptide antagonist. Evidence for a composite ligand-binding site and a short interdomain separation" BIOCHEMISTRY, vol. 37, no. 11, 17 March 1998, EASTON, PA US, pages 3612-3622, XP002124882

D4: FAUCHÈRE E.A.: "Amino acid sidechain parameters for correlation studies in biology and pharmacology" INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH, vol. 32, no. 4, October 1988, COPENHAGEN DK, pages 269-278, XP000857464 cited in the application

1. This application concerns peptides that inhibit the binding of the protease, uPA, to its cognate receptor, uPAR. The peptides are synthesised by methods of solid peptide synthesis and comprise also non-natural synthetic amino acids.

D1 describes peptide antagonist of uPA that are synthetically synthesized and that compete with the amino terminal fragment (ATF) of uPA for uPAR binding with IC<sub>50</sub> values in the low nanomolar range.

D2 discloses high-affinity uPAR antagonists discovered using bacteriophage peptide display techniques. The most potent peptide, clone 20, served as a starting point for the development of the peptide antagonists of D1 and of the peptides claimed in the application. D3 by the applicants studies in detail the molecular aspects of the interaction between a clone 20-derived peptide and uPAR by amino acid substitutions and plasmon resonance, among other techniques. D3 delineates the composite binding region of uPAR and defines residues in the peptide antagonist critical for binding.

D4 discloses physicochemical calculations of the contribution of natural and non-natural amino acid residues to particular physicochemical properties of peptides.

## **2. Novelty of claim 1**

The prior art does not disclose a peptide as defined in claim 1. The subject-matter of independent claim 1 and dependent claims 2-37 therefore is novel (Article 33(2) PCT).

## **3. Obviousness of claim 1**

D1 constitutes the closest prior art, since it describes nona- and decapeptide antagonists of uPA that inhibit its binding in the low nanomolar range. These peptides are improved derivatives of the original chiron clone 20 high-affinity antagonist disclosed in D2. In view of D1, the underlying technical problem of the application is to provide alternative peptide antagonists of uPA.

This technical problem is solved by introducing a non-natural amino acid,  $\beta$ -cyclohexyl-alanine (Cha), in relative position 3 of the minimal antagonist, AE68 (AE68

is a truncated clone 20 peptide that retains antagonistic activity. This peptide is disclosed in D3 and serves in the application as a starting point for the synthesis of higher affinity peptides). All peptides synthesized by the applicants contain a Cha residue at the position neighboring N-terminally the essential Phe residue (Phe at position 4 of AE68, explained in detail on page 40, line 5 to page 42, line 7 of the application; also D3, figure 10). Apparently, this substitution is critical for the improved kinetics of binding of the peptides dCha, AE100, AE108, AE105, AE110, and AE114, which display reduced off-rates compared to an optimised chiron clone 20 peptide.

The peptide sequences specified in D1 do not contain a Cha residue N-terminally to the essential Phe residue. D1 lists on pages 4-6 non-conventional amino acids, among them Cha (page 4, left column line 31), that may be introduced in the peptide inhibitors without specifying at which position. D3 defines critical residues of a clone 20-derived minimal antagonist decapeptide which are essential for binding to uPAR. Figures 3 and 4 show alanine replacement experiments where the alanine-substituted clone 20 derivative competes with ATF for binding to uPAR. Critical for binding are the residues Leu, Phe, Leu or Trp at relative positions 2, 4, 8, and 9, respectively. Substitution of Asn at relative position 3 causes only a moderate effect and is therefore considered not to be essential for good antagonistic properties (page 3615, right column, second paragraph; page 3619, right column; figures 3, 4, 5, and 10). It is though exactly this Asn residue that is replaced by Cha in the application (This can be readily seen by aligning the peptides on page 36 of the application with peptide antagonist AE68 which is the clone 20 minimal decapeptide antagonist). Even when combining the teachings of D1 and D3, the skilled person would not be guided to replace the Asn in position 3 of the peptide AE68. Moreover, D1 provides a list of possible non-natural amino acids, but does not indicate that Cha N-terminally to the essential Phe would be of particular usefulness. D4 examines also some physicochemical properties of Cha residues (table 2) and states that the listed properties may be useful for quantitative investigations of peptide drug-receptor interactions (page 277, right column, last paragraph), but these indications are general and not related to uPAR peptide antagonists.

Thus, independent claim 1 and dependent claims 2-37 are not obvious and fulfil the requirements of Article 33(3) PCT. For clarity, see also VIII.

**4. Novelty and Inventive Step of claims 38-40**

Claims 38-40 are inventive in so far as they refer to the products that are novel and inventive (Article 33(3) PCT).

**5. Industrial applicability of claim 40**

For the assessment of the present claim 40 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

**Re Item VIII**

Certain observations on the international application

1. Claim 1 is very broad. The synthesized peptides listed on page 36 of the description constitute only a very small fraction of all possible peptides falling under the protection of claim 1. Thus, claim 1 is partly not supported by the description. The vague and imprecise term "optionally substituted" ring makes it impossible to define exactly the subject-matter of claim 1. Besides, it is very probable that a completely unrestricted choice of the substituents of the rings in the side chain of amino acids X2 would abolish the antagonistic properties of the claimed peptide. Claim 1 in its present form does therefore not satisfy Article 6, PCT. In fact, only product claims 25 and 36 are fully supported by the description.
2. Claims 13 and 14 refer to a hydrophobicity constant without defining this constant and referring to the description. According to Rule 6.2a) PCT, such references to the description are not allowed.
3. Claims 17-25 refer to peptides, but contain also peptoid sequences. Peptoids are not peptides. Peptoids are synthetic molecules that are "peptide-like" polymers, as stated in the application itself (page 22, lines 11-12). Said claims are therefore unclear (Article 6 PCT).

4. The pages 1-9 furnished with letter of 2 November, 1999 containing sequence protocols are not part of the application (Rule 13ter 1f) PCT) and the claims should therefore not refer to SEQ ID numbers.

Moreover, SEQ ID No:1 is used for two completely different molecules in claim 25. This is unclear (Article 6 PCT). In claim 36, SEQ ID No:4 is used for three different branched peptides. Besides, SEQ ID No:4 is already given to an unbranched nonapeptide in claim 25. For these reasons, the applicants should omit SEQ ID numbers.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 9/72, G01N 33/68, 33/566, A61K 38/49, G01N 33/50</b>		<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/01802</b>
		<b>(43) International Publication Date:</b> 13 January 2000 (13.01.00)	
<b>(21) International Application Number:</b> PCT/DK99/00377 <b>(22) International Filing Date:</b> 1 July 1999 (01.07.99)  <b>(30) Priority Data:</b> PA 1998 00874 1 July 1998 (01.07.98) DK  <b>(71) Applicant (for all designated States except US):</b> CANCERFORSKNINGSFONDEN AF 1989 (FONDEN TIL FREMME AF EKSPERIMENTEL CANCERFORSKNING) [DK/DK]; Michael Rostock, Vester Voldgade 90, DK-1552 Copenhagen V (DK).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> PLOUG, Michael [DK/DK]; Nørrebrogade 63, 2. th., DK-2200 Copenhagen N (DK). ØSTERGAARD, Søren [DK/DK]; Borups Allé 235D, st.tv., DK-2400 Copenhagen NV (DK). HOLM, Arne [DK/DK]; Margrethevej 19, DK-2840 Holte (DK). HOLST-HANSEN, Claus [DK/DK]; Hostrupvej 5, 1.tv., DK-1950 Frederiksberg C (DK). STEPHENS, Ross, W. [DK/DK]; Broholms Allé 2. th., DK-2920 Charlottenlund (DK). DANØ, Keld [DK/DK]; L. E. Bruuhnsvej 20, DK-2920 Charlottenlund (DK).		<b>(74) Agent:</b> PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annæ Plads 11, P.O. Box 3007, DK-1021 Copenhagen K (DK).  <b>(81) Designated States:</b> AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>	
<b>(54) Title:</b> PEPTIDE ANTAGONISTS OF THE HUMAN UROKINASE RECEPTOR AND METHOD FOR SELECTING THEM			
<b>(57) Abstract</b>  A novel set of inhibitors of the binding interaction between human urokinase plasminogen activator (uPA) and its cell surface receptor (uPAR) has been developed. The inhibitors comprise of peptide fragments, monomeric or in multiple copies attached to a common scaffold, in which the amino acid sequence may include uncommon substituted amino acids to partially comprise of peptoid sequences. The present invention also relates to the use of such peptides in therapy, in particular for the treatment of cancer, having developed a modified non-human mammalian receptor to which the novel inhibitors are antagonistic.			

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## PEPTIDE ANTAGONISTS OF THE HUMAN UROKINASE RECEPTOR

## FIELD OF THE INVENTION

- 5 The present invention relates to novel peptide inhibitors of the binding interaction between human urokinase plasminogen activator (in the following referred to as uPA) and its cell surface receptor (in the following referred to as uPAR). The present invention also relates to such peptides for use in therapy.

## 10 GENERAL BACKGROUND

- The use of inhibitors of the binding interaction between uPA and uPAR provide a method for preventing or counteracting localised extracellular proteolytic activity in a mammal, in particular a human, by preventing the binding of a receptor-binding form of uPA to uPAR in  
15 the mammal and thereby reducing the ability of uPA to convert plasminogen into plasmin. This mechanism is *inter alia* described in N. Behrendt et al. (1995) *Biol. Chem. Hoppe-Seyler*, 376:269-279 and in WO 90/12091. While the binding of uPA is known to be necessary for optimal performance of cell-surface proteolysis, blocking of uPA may also have other anti-tumour effects in the context of cell signalling, cell adhesion and migration.  
20 Such alternative mechanisms are e.g. suggested in Min et al., *Cancer Research*, 56: 2428-2433 (1996).

- Peptide inhibitors of the uPA/uPAR interaction have previously been described (see R.J. Goodson et al. (1994) *Proc. Nat. Acad. Sci. U.S.A.* 91:7129-7133; M. Bürgle et al. (1997)  
25 *Biol. Chem.* 378: 231-237; U.S. patent No. 5,656,726; WO 97/35969 and WO 97/24453).

- Thus, Goodson et al. disclosed nineteen linear 15-mers comprising of two relatively short conserved subsequences: LWXXAr (Ar = Y, W, F or H) and XFXXYLW, neither of which are found in uPA or its receptor (uPAR). The peptides were tested in a uPAR binding  
30 assay, wherein the most potent inhibitor (the so-called "Clone 20": AEPMPHSLNFSQYLWYT) showed an apparent inhibition constant at 10 nM for the uPAR-ATF interaction.

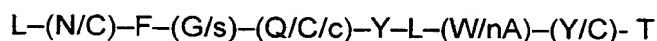
- Bürgle, et al. studied the ability of synthetic peptides derived from the uPAR binding region  
35 of uPA (i.e. comprising the amino acids 16-32 of uPA) to inhibit the uPA/uPAR interaction.



Moreover, various disulfide-bridged cyclic forms of the above-mentioned peptide were tested and it was found that the cyclo<sup>19,31</sup>uPA<sub>19-31</sub> was a relatively potent inhibitor of the uPA/uPAR interaction.

- 5 WO 97/35969 (Chiron) describes a number of peptides capable of binding to uPAR thereby inhibiting the binding of an integrin and vitronectin.

WO 97/05257 (Chiron) relates *inter alia* to polypeptides and analogues thereof capable of binding to uPAR and thereby inhibit the receptor binding activity of uPA. The peptides  
10 studied all comprised the following sequence:



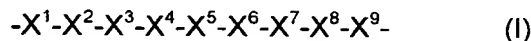
wherein capital letters designate L-amino acids, lower case letters designate D-amino  
15 acids and nA denotes 1-naphthylalanine. It was found that inclusion of D-serine in the position next to the fixed phenylalanine in most cases caused a 2-5 fold decrease in the obtained IC<sub>50</sub> value.

#### SUMMARY OF THE INVENTION

20

The present invention provides peptides having considerably higher affinity for uPAR, lower off-rates, high potency in a cell-binding system, and demonstrated biological stability.

Thus, in a first aspect the present invention relates to a peptide comprising at least one  
25 peptide fragment of the general formula I:

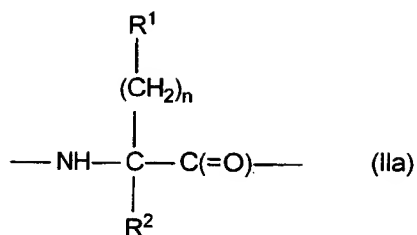


wherein

30

X<sup>1</sup>, X<sup>5</sup>, X<sup>6</sup>, X<sup>7</sup> and X<sup>9</sup> are independently selected from amino acids and X<sup>1</sup> is the N-terminal amino acid of the fragment and X<sup>9</sup> is the C-terminal amino acid of the fragment;

X<sup>2</sup> is selected from the group consisting of amino acids of the general formula IIa

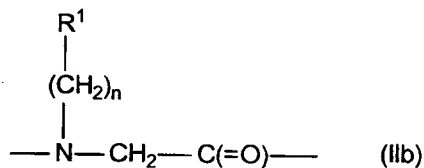


wherein n is an integer in the range from 0 to 3; R<sup>1</sup> is selected from the group consisting of optionally substituted five-, six- and seven-membered non-aromatic rings; R<sup>2</sup> is selected  
 5 from the group consisting of hydrogen and C<sub>1-4</sub>-alkyl; or,

R<sup>1</sup> and R<sup>2</sup> together with the carbon atom to which they are bound form an optionally substituted cyclopentyl, cyclohexyl, cycloheptyl or decahydronaphthalenyl ring;

10 and

N-substituted amino acids of the general formula IIb



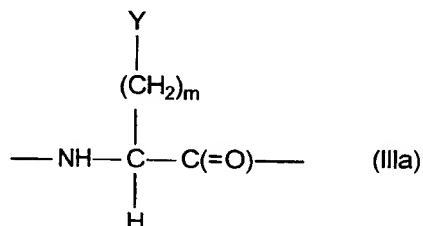
15

wherein n and R<sup>1</sup> are as defined above.

X<sup>3</sup> and X<sup>8</sup> are each independently selected from the group consisting of amino acids having hydrophobic side chains and amino acids having hydrophobic N-substituents;

20

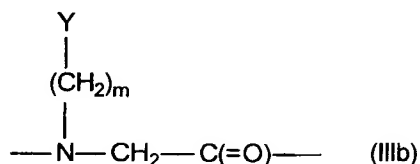
X<sup>4</sup> is selected from the group consisting of amino acids of the general formula IIIa



wherein m is an integer in the range from 1 to 3, and Y is selected from the group consisting of OH, SH, NH<sub>2</sub>, CONH<sub>2</sub>, COOH and OPO<sub>3</sub>H;

5 and

N-substituted amino acids of the general formula IIIb



10 wherein m and Y are as defined above;

Moreover the present invention relates to *in vitro* and *in vivo* methods for selecting peptide antagonists which are suitable for preventing or counteracting localized extracellular proteolytic activity of plasmin in a human.

15

In further aspects the present invention relates to a peptide as defined above for use as a medicament, to the use of the peptide defined above for the manufacture of a medicament for treatment of cancer, and to a pharmaceutical composition comprising the peptide of the invention.

20

## DETAILED DESCRIPTION OF THE INVENTION

In the present context the term "C<sub>1-7</sub>-alkyl" used alone or as part of another group designates a linear, branched or cyclic saturated hydrocarbon group having from one to  
25 seven carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, cyclobutyl, pentyl, isopentyl, neopentyl, cyclopentyl, hexyl, cyclohexyl, heptyl and cycloheptyl. Analogously, the term "C<sub>1-4</sub>-alkyl" used alone or as part of another group designates a linear, branched saturated hydrocarbon group having from one to four carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl and tert-butyl.

30

In the present context the term "C<sub>2-8</sub>-alkenyl" is intended to mean a linear, branched or cyclic hydrocarbon group having from two to eight carbon atoms and containing one or

more double bonds. Examples of C<sub>2-8</sub>-alkenyl groups include olefins such as allyl, homo-allyl, vinyl, crotyl, butenyl, pentenyl, hexenyl, heptenyl and octenyl. Examples of C<sub>2-8</sub>-alkenyl groups with more than one double bond include butadienyl, pentadienyl, hexadienyl, heptadienyl, hexatrienyl, heptatrienyl and octatrienyl groups as well as  
5 branched forms of these. Preferred examples of C<sub>2-8</sub>-alkenyls are vinyl, allyl and butenyl.

In the present context the term "C<sub>2-8</sub>-alkynyl" is intended to mean linear, branched or cyclic hydrocarbon groups containing from two to eight carbon atoms and containing one or more triple bonds. Examples of C<sub>2-8</sub>-alkynyl groups include acetylene, propynyl, butynyl,  
10 pentynyl, hexynyl, heptynyl and octynyl groups as well as branched forms of these.

When used herein the term "C<sub>1-7</sub>-alkoxy" is intended to mean C<sub>1-7</sub>-alkyl-oxy such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, pentoxy, isopentoxy, neopentoxy, hexoxy and heptoxy. In a similar way, when used herein  
15 the term "C<sub>1-4</sub>-alkoxy" is intended to mean C<sub>1-4</sub>-alkyl-oxy such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy and tert-butoxy.

In the present context the term "aryl" used alone or as part of another group is intended to mean an aromatic carbocyclic ring or ring system, such as phenyl, naphthyl, anthracyl,  
20 phenanthracyl, pyrenyl, benzopyrenyl, fluorenyl, and xanthenyl, preferably phenyl.

The term "heteroaryl" is intended to mean an aryl group where one or more carbon atoms have been replaced with heteroatoms such as nitrogen, sulphur, and/or oxygen atoms. Examples of such heteroaryl groups are oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, pyrrolyl,  
25 imidazolyl, pyrazolyl, pyridinyl, pyrazinyl, pyridazinyl, piperidinyl, coumaryl, furyl, quinolyl, indolyl, benzopyrazolyl, and phenoxazolyl.

In the present context the term "five-, six- and seven-membered non-aromatic ring" is intended to cover five-, six- and seven-membered rings comprising carbon atoms only  
30 (carbocyclic) or carbon atoms together with from 1 to 3 heteroatoms (heterocyclic), wherein the heteroatoms are independently selected from oxygen, sulphur, and nitrogen. Such rings may contain no unsaturated bonds or may contain one or more unsaturated bonds, however, if present, situated in such a way that an aromatic  $\pi$ -electron system does not arise.

- Examples of preferred "five-, six- and seven-membered non-aromatic rings" are cyclopentane, cyclopentene, cyclopentadiene, cyclohexane, cyclohexene, 1,3-cyclohexadiene, 1,4-cyclohexadiene, cycloheptane, cycloheptene, 1,2-cycloheptadiene, 1,3-cycloheptadiene, 1,4-cycloheptadiene, 1,3,5 cycloheptatriene, 2*H*-thiopyran, 3*H*-thiopyran, 4*H*-thiopyran, tetrahydrothiopyran, 2*H*-pyran, 4*H*-pyran, tetrahydropyran, piperidine, 1,2-dithiin, 1,2-dithiane, 1,3-dithiin, 1,3-dithiane, 1,4-dithiin, 1,4-dithiane, 1,2-dioxin, 1,2-dioxane, 1,3-dioxin, 1,3-dioxane, 1,4-dioxin, 1,4-dioxane, piperazine, 1,2-oxathiin, 1,2-oxathiane, 4*H*-1,3-oxathiin, 1,3-oxathiane, 1,4-oxathiin, 1,4-oxathiane, 2*H*-1,2-thiazine, tetrahydro-1,2-thiazine, 2*H*-1,3-thiazine, 4*H*-1,3-thiazine, 5,6-dihydro-4*H*-thiazine, 4*H*-1,4-thiazine, tetrahydro-1,4-thiazine, 2*H*-1,2-oxazine, 4*H*-1,2-oxazine, 6*H*-1,2-oxazine, 2*H*-1,3-oxazine, 4*H*-1,3-oxazine, 4*H*-1,4-oxazine, morpholine, trioxane, 4*H*-1,2,3-trithiin, 1,2,3-trithiane, 1,3,5-trithiane, hexahydro-1,3,5-triazine, tetrahydrothiophene, tetrahydrofuran, pyrroline, pyrrolidine, pyrrolidone, pyrrolidione, pyrazoline, pyrazolidine, imidazoline, imidazolidine, 1,2-dioxole, 1,2-dioxolane, 1,3-dioxole, 1,3-dioxolane, 3*H*-1,2-dithiole, 1,2-dithiolane, 1,3-dithiole, 1,3-dithiolane, isoxazoline, isoxazolidine, oxazoline, oxazolidine, thiazoline, thiozolidine, 3*H*-1,2-oxathiole, 1,2-oxathiolane, 5*H*-1,2-oxathiole, 1,3-oxathiole, 1,3-oxathiolane, 1,2,3-trithiole, 1,2,3-trithiolane, 1,2,4-trithiolane, 1,2,3-trioxole, 1,2,3-trioxolane, 1,2,4-trioxolane, 1,2,3-triazoline and 1,2,3-triazolidine.
- 20 The term "halogen" includes fluorine, chlorine, bromine and iodine.

- In the present context, i.e. in connection with the terms "aryl", "heteroaryl", "carbocyclic", "cyclopentyl", "cyclohexyl", "cycloheptyl", "decahydronaphthalenyl", "five-, six- and seven-membered non-aromatic ring", "C<sub>1-7</sub>-alkyl", "C<sub>1-7</sub>-alkoxy", "C<sub>2-8</sub>-alkenyl", and "C<sub>2-8</sub>-alkynyl", the term "optionally substituted" is intended to mean that the group in question may be substituted one or several times, such as 1 to 5 times, preferably 1 to 3 times, with one or more groups selected from C<sub>1-7</sub>-alkyl, such as C<sub>1-4</sub>-alkyl, C<sub>1-7</sub>-alkoxy, such as C<sub>1-4</sub>-alkoxy, oxo (which may be represented in the tautomeric enol form), carboxy, amino, hydroxy (which when present in an enol system may be represented in the tautomeric keto form), nitro, sulphonyl, sulphonyl, C<sub>1-7</sub>-carboxy, C<sub>1-7</sub>-alkoxycarbonyl, C<sub>1-7</sub>-alkylcarbonyl, formyl, aryl, aryloxy, aryloxycarbonyl, arylcarbonyl, heteroaryl, amino, mono- and di(C<sub>1-7</sub>-alkyl)amino; carbamoyl, mono- and di(C<sub>1-7</sub>-alkyl)aminocarbonyl, amino-C<sub>1-7</sub>-alkyl-aminocarbonyl, mono- and di(C<sub>1-7</sub>-alkyl)amino-C<sub>1-7</sub>-alkyl-aminocarbonyl, C<sub>1-7</sub>-alkylcarbonylamino, cyano, guanidino, carbamido, C<sub>1-7</sub>-alkanoyloxy, sulphonyl, C<sub>1-7</sub>-alkylsulphonyloxy, nitro, sulphonyl, dihalogen-C<sub>1-4</sub>-alkyl, trihalogen-C<sub>1-4</sub>-alkyl, halogen, where aryl and heteroaryl

representing substituents may be substituted 1-3 times with C<sub>1-4</sub>-alkyl, C<sub>1-4</sub>-alkoxy, nitro, cyano, hydroxy, amino or halogen. Preferred examples are hydroxy, C<sub>1-4</sub>-alkyl, C<sub>1-4</sub>-alkoxy, carboxy, C<sub>1-4</sub>-alkoxycarbonyl, C<sub>1-4</sub>-alkylcarbonyl, aryl, amino, mono- and di(C<sub>1-4</sub>-alkyl)amino, and halogen, wherein aryl may be substituted 1-3 times with C<sub>1-4</sub>-alkoxy, nitro, cyano, amino or halogen.

In the present context the term "amino acid", unless otherwise stated, is intended to cover the common  $\alpha$ -amino acids, i.e. L-alanine (A), L-valine (V), L-leucine (L), L-isoleucine (I), L-methionine (M), L-phenylalanine (F), L-tryptophan (W), L-proline (P), glycine (G), L-serine (S), L-threonine (T), L-cysteine (C), L-tyrosine (Y), L-asparagine (N), L-glutamine (Q), L-lysine (K), L-arginine (R), L-histidine (H), L-aspartic acid (D) and L-glutamic acid (E).

The term "amino acid", however, is also intended to cover unconventional  $\alpha$ -amino acids such as D-amino acids,  $\alpha$ -alkylated amino acids, such as  $\alpha$ -methylated amino acids and N-substituted amino acids, in particular N-substituted glycines.

Examples of such unconventional amino acids are D-alanine (a), D-valine (v), D-leucine (l), D-isoleucine (i), D-methionine (m), D-phenylalanine (f), D-tryptophan (w), D-proline (p), D-serine (s), D-threonine (t), D-cysteine (c), D-tyrosine (y), D-asparagine (n), D-glutamine (q), D-lysine (k), D-arginine (r), D-histidine (h), D-aspartic acid (d), D-glutamic acid (e), L-norleucine, L-homophenylalanine, D-ornithine, L-ethylglycine, penicillamine, cyclohexylalanine, 1-aminocyclopropane-1-carboxylate, L- $\alpha$ -methylalanine, L- $\alpha$ -methylasspartic acid, L- $\alpha$ -methylphenylalanine, L- $\alpha$ -isoleucine, L- $\alpha$ -methylleucine, L- $\alpha$ -methylassparagine, L- $\alpha$ -methylglutamine, L- $\alpha$ -methylserine, L- $\alpha$ -methylvaline, L- $\alpha$ -methylnorleucine, L- $\alpha$ -methylnorvaline, L- $\alpha$ -methylethylglycine,  $\alpha$ -methylaminoisobutyric acid,  $\alpha$ -methylpenicillamine,  $\alpha$ -methylcyclohexylalanine, D- $\alpha$ -methylalanine, D- $\alpha$ -methylcysteine, D- $\alpha$ -methylglutamic acid,  $\alpha$ -amino butyric acid, L-norvaline,  $\alpha$ -aminoisobutyric acid, L- $\alpha$ -*t*-butylglycine,  $\alpha$ -naphthylalanine, cyclopentylalanine, 1-aminonorbornyl-1-carboxylate, L- $\alpha$ -methylcysteine, L- $\alpha$ -methylglutamic acid, L- $\alpha$ -methylhistidine, L- $\alpha$ -methyllysine, L- $\alpha$ -methylmethionine, L- $\alpha$ -methylproline, L- $\alpha$ -methylarginine, L- $\alpha$ -methylthreonine, L- $\alpha$ -methyltryptophan, L- $\alpha$ -methylornithine,  $\alpha$ -amino- $\alpha$ -methylbutyric acid, L- $\alpha$ -methylhomophenylalanine, L- $\alpha$ -methyl-*t*-butylglycine,  $\alpha$ -methyl- $\alpha$ -naphthylalanine,  $\alpha$ -methylcyclopentylalanine, D- $\alpha$ -methylasspartic acid, D- $\alpha$ -methylphenylalanine, D- $\alpha$ -methylhistidine, D- $\alpha$ -methyllysine, D- $\alpha$ -methylmethionine, D- $\alpha$ -methylproline, D- $\alpha$ -methylarginine, D- $\alpha$ -methylthreonine, D- $\alpha$ -

methyltryptophan, D- $\alpha$ -methylisoleucine, D- $\alpha$ -methylleucine, D- $\alpha$ -methy lasparagine, D- $\alpha$ -  
 methylglutamine, D- $\alpha$ -methylserine, D- $\alpha$ -methylvaline, D- $\alpha$ -methyltyrosine, L-N-  
 methylalanine, L-N-methylphenylalanine, L-N-methylisoleucine, L-N-methylleucine, L-N-  
 methy lasparagine, L-N-methylglutamine, L-N-methylserine, L-N-methylvaline, L-N-  
 5 methyltyrosine, L-N-methylnorleucine, L-N-methylnorvaline, N-methylcyclopentylalanine, N-  
 methylpenicillamine, N-methylaminoisobutyric acid, D-N-methylalanine, D-N-  
 methylcysteine, D-N-methylglutamic acid, D-N-methylhistidine, D-N-methyllysine, D-N-  
 methylmethionine, D-N-methylproline, D-N-methylarginine, D-N-methylthreonine, D-N-  
 methyltryptophan, N-methylglycine (sarcosine), L-N-methylcysteine, L-N-methylglutamic  
 10 acid, L-N-methylhistidine, L-N-methyllysine, L-N-methylmethionine, N-methylcyclohexyl-  
 alanine, L-N-methylarginine, L-N-methylthreonine, L-N-methyltryptophan, L-N-  
 methylornithine, N-amino- $\alpha$ -methylbutyric acid, L-N-methylhomophenylalanine, N-methyl-  
 $\alpha$ -naphthylalanine, D-N-methylornithine, D-N-methy laspartic acid, D-N-  
 methylphenylalanine, D-N-methylisoleucine, D-N-methylleucine, D-N-methy lasparagine, D-  
 15 N-methylglutamine, D-N-methylglutamine, D-N-methylserine, D-N-methylvaline, D-N-  
 methyltyrosine, and N-substituted glycines such as, e.g., L-N-methylethylglycine, N-(2-  
 carboxyethyl)glycine, N-(imidazolethyl)glycine, N-(4-aminobutyl)glycine, N-(2-methylthio-  
 ethyl)glycine, N-(carbamylmethyl)glycine, N-(1-methylethyl)glycine, L-N-methyl-*t*-  
 butylglycine, N-(carboxymethyl)glycine, N-ethylglycine, N-benzylglycine, N-(1-  
 20 methylpropyl)glycine, N-(2-methylpropyl)glycine, N-(hydroxyethyl)glycine, N-(2-  
 carbamylethyl)glycine, N-(3-guanidinopropyl)glycine, N-(3-indolylethyl)glycine, N-(1-  
 hydroxyethyl)glycine, N-(3-aminopropyl)glycine, N-cyclobutylglycine, N-cycloheptylglycine,  
 N-cyclodecylglycine, N-cyclododecylglycine, N-(3,3-diphenylpropyl)glycine, N-(N-(2,2-  
 diphenylethyl)carbamylmethyl)glycine), N-(N-(3,3-diphenylpropyl)carbamylmethyl)glycine),  
 25 N-(2-(4-methoxy)-phenyl)glycine, 1-carboxy-1-(2,2-diphenylethylamino)cyclopropane, N-(2-  
 aminoethyl)glycine, N-(*p*-hydroxyphenethyl)glycine, N-(thiomethyl)glycine, N-  
 cyclopropylglycine, N-cyclohexylglycine, N-methylcyclohexylglycine, N-cyclooctylglycine,  
 N-cycloundecylglycine and N-(2,2-diphenylethyl)glycine, N-(2,3-dimethoxybenzyl)glycine,  
 N-(naphthyl)glycine, N-(methylnaphthalyl)glycine, N-(indanyl)-glycine, N-  
 30 (diphenylethyl)glycine and N-(2-methoxyethyl)glycine.

Thus, in the present context the term "substituted" as part of the terms "N-substituted  
 glycines" and "N-substituted amino acids" is intended to mean the case where an amine  
 hydrogen is replaced by a hydrocarbon group such as optionally substituted C<sub>1-7</sub>-alkyl,  
 35 optionally substituted C<sub>2-8</sub>-alkenyl, and optionally substituted C<sub>2-8</sub>-alkynyl groups. The term

"substituent" refers to the group that has replaced the hydrogen on the amine nitrogen. Analogously, the term "side chain", when used herein, refers to the actual substituent (or substituents) on the  $\alpha$ -carbon atom. Thus, the term "side chain" does not refer to substituents present on the amine nitrogen atom of the amino acid, cf. above.

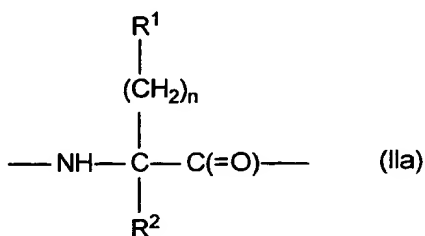
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Thus, the amino acids  $X^1$ ,  $X^5$ ,  $X^6$ ,  $X^7$  and  $X^9$  in the peptide fragment of the general formula I, as defined above, are independently selected from such amino acids as mentioned above. Preferably, however, the amino acids  $X^1$ ,  $X^5$ ,  $X^6$ ,  $X^7$  and  $X^9$  are independently selected from the group consisting of L-alanine, L-valine, L-leucine, L-isoleucine, L-methionine, L-phenylalanine, L-tryptophan, L-proline, glycine, L-serine, L-threonine, L-cysteine, L-tyrosine, L-asparagine, L-glutamine, L-lysine, L-arginine, L-histidine, L-aspartic acid, L-glutamic acid, D-alanine, D-valine, D-leucine, D-isoleucine, D-methionine, D-phenylalanine, D-tryptophan, D-proline, D-serine, D-threonine, D-cysteine, D-tyrosine, D-asparagine, D-glutamine, D-lysine, D-arginine, D-histidine, D-aspartic acid and D-glutamic acid as well as the N-substituted glycine analogues of the above-mentioned amino acids. Thus, as will be understood by the person skilled in the art, the term "N-substituted glycine analogues" means that the N-substituent on the N-substituted glycine is similar (and, in some cases identical) to the side chain of the amino acid in question. This may be further illustrated by the following example; The "N-substituted glycine analogue" to alanine is N-methyl-glycine as the methyl group has been "transposed" from the  $\alpha$ -carbon to the amine part of the molecule. Accordingly, the amino acid alanine may be considered as an  $\alpha$ -methyl substituted glycine molecule.

As will be apparent from the examples provided herein, the amino acid  $X^2$  in the peptide fragment of the general formula I, as defined above, is of utmost importance for efficient binding of the peptides of the invention to the uPAR. Without being limited thereto, it is presently believed that some aliphatic ring system must be present at the side chain of the  $X^2$  amino acid (or, in an analogous way, some aliphatic ring system must be present on the N-substituent of the N-substituted  $X^2$  amino acid) in order to enhance the binding of the peptide to uPAR.

Thus, in one interesting embodiment of the invention  $X^2$  is an amino acid of the general formula IIa





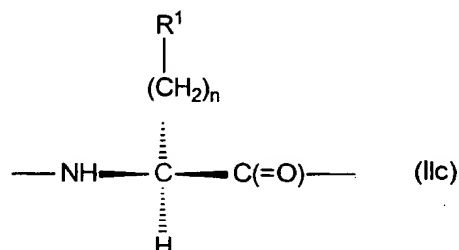
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wherein n is an integer in the range from 0 to 3, such as 1, 2 or 3, preferably 1 or 2. R<sup>1</sup> is selected from the group consisting of optionally substituted five-, six- and seven-membered non-aromatic rings such as cyclopentane, cyclopentene, cyclopentadiene, cyclohexane, cyclohexene, 1,3-cyclohexadiene, 1,4-cyclohexadiene, cycloheptane, cycloheptene, 1,2-cycloheptadiene, 1,3-cycloheptadiene, 1,4-cycloheptadiene, 1,3,5 cycloheptatriene, 2H-thiopyran, 3H-thiopyran, 4H-thiopyran, tetrahydrothiopyran, 2H-pyran, 4H-pyran, tetrahydropyran, piperidine, 1,2-dithiin, 1,2-dithiane, 1,3-dithiin, 1,3-dithiane, 1,4-dithiin, 1,4-dithiane, 1,2-dioxin, 1,2-dioxane, 1,3-dioxin, 1,3-dioxane, 1,4-dioxin, 1,4-dioxane, piperazine, 1,2-oxathiin, 1,2-oxathiane, 4H-1,3-oxathiin, 1,3-oxathiane, 1,4-oxathiin, 1,4-oxathiane, 2H-1,2-thiazine, tetrahydro-1,2-thiazine, 2H-1,3-thiazine, 4H-1,3-thiazine, 5,6-dihydro-4H-thiazine, 4H-1,4-thiazine, tetrahydro-1,4-thiazine, 2H-1,2-oxazine, 4H-1,2-oxazine, 6H-1,2-oxazine, 2H-1,3-oxazine, 4H-1,3-oxazine, 4H-1,4-oxazine, morpholine, trioxane, 4H-1,2,3-trithiin, 1,2,3-trithiane, 1,3,5-trithiane, hexahydro-1,3,5-triazine, tetrahydrothiophene, tetrahydrofuran, pyrroline, pyrrolidine, pyrrolidone, pyrrolidione, pyrazoline, pyrazolidine, imidazoline, imidazolidine, 1,2-dioxole, 1,2-dioxolane, 1,3-dioxole, 1,3-dioxolane, 3H-1,2-dithiole, 1,2-dithiolane, 1,3-dithiole, 1,3-dithiolane, isoxazoline, isoxazolidine, oxazoline, oxazolidine, thiazoline, thiozolidine, 3H-1,2-oxathiole, 1,2-oxathiolane, 5H-1,2-oxathiole, 1,3-oxathiole, 1,3-oxathiolane, 1,2,3-trithiole, 1,2,3-trithiolane, 1,2,4-trithiolane, 1,2,3-trioxole, 1,2,3-trioxolane, 1,2,4-trioxolane, 1,2,3-triazoline and 1,2,3-triazolidine.

R<sup>2</sup> is hydrogen or C<sub>1-4</sub>-alkyl, such as methyl or ethyl, but preferably hydrogen.

As indicated above,  $X^2$  may be an amino acid of the general formula IIa. However, in a preferred embodiment of the invention, the  $X^2$  amino acid is restricted to a specific stereochemistry around the asymmetric  $\alpha$ -carbon atom. Therefore,  $X^2$  preferably has the structure as indicated in the general formula IIc:

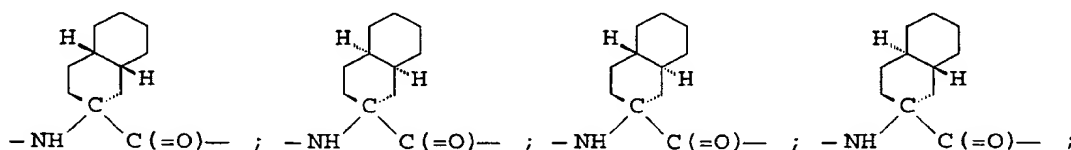
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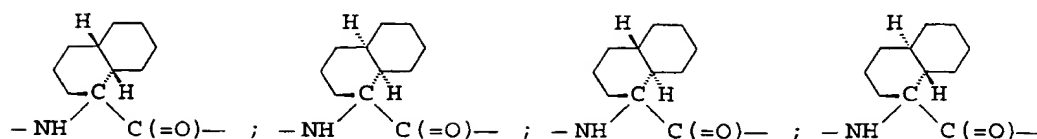


wherein  $n$  is 1 or 2 and  $R^1$  is as defined above.

In a particular interesting embodiment of the invention  $R^1$  is cyclopentyl, cyclohexyl or  
 10 cycloheptyl and  $n$  is preferably 1, i.e. specific examples of amino acids which are especially suitable in the  $X^2$  position are  $\beta$ -cyclopentyl-L-alanine,  $\beta$ -cyclohexyl-L-alanine and  $\beta$ -cycloheptyl-L-alanine, in particular  $\beta$ -cyclohexyl-L-alanine.

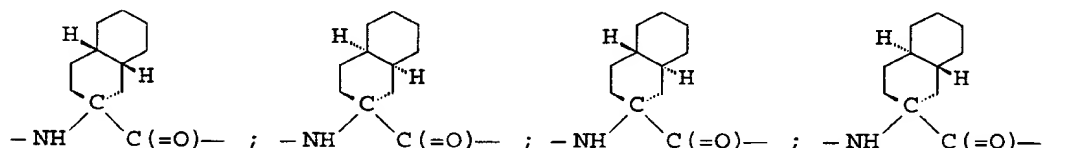
In another interesting embodiment of the invention,  $R^1$  and  $R^2$  together with the  $\alpha$ -carbon  
 15 atom to which they are attached form an optionally substituted carbocyclic ring, preferably a cyclopentyl, cyclohexyl or cycloheptyl ring. Furthermore,  $R^1$  and  $R^2$  together with the  $\alpha$ -carbon atom to which they are attached may form a decahydronaphthalenyl ring. It should be understood that the decahydronaphthalenyl ring, when attached to the amino acid skeleton, may appear in several different isomeric forms (*vide infra*). First of all, when the  
 20 decahydronaphthalenyl group is formed together with the  $\alpha$ -carbon atom of the  $X^2$  amino acid, two different structural isomers are possible (*vide infra*). In addition to that, each of the structural isomers may exist in four different diastereomeric forms as well as the mirror images thereof. Thus, the decahydronaphthalenyl ring, when attached to the amino acid skeleton, may appear in a total of sixteen different isomeric forms which are all within the  
 25 scope of the invention. In other words, in an interesting embodiment of the invention, the amino acid  $X^2$  has the following structures:





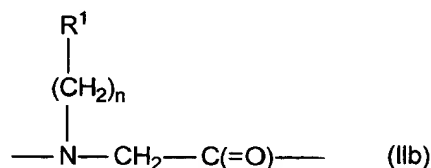
as well as the mirror image stereoisomers thereof.

- 5 As mentioned above, and as will be understood from the examples provided herein,  $\beta$ -cyclohexyl-L-alanine is especially suitable as the  $X^2$  amino acid. Accordingly, although a total of sixteen different isomers are possible for the decahydronaphthalenyl ring system, it is envisaged that the following structures (as well as the mirror images thereof) are particular suitable as the  $X^2$  amino acid since these structures resemble the  $\beta$ -cyclohexylalanine structure:



- The structures shown above (as well as the mirror images thereof) may be considered as being  $\beta$ -cyclohexylalanine analogues, wherein the cyclohexyl ring of  $\beta$ -cyclohexylalanine has been "locked" in specific conformations.

In a further interesting embodiment of the invention  $X^2$  is an N-substituted amino acid (or, more precisely, an N-substituted glycine) of the general formula IIb



wherein  $R^1$  and  $n$  are as defined above.

As will be understood from the examples provided herein, in several cases similar performance by the peptide is achieved whether the side chain is present on the  $\alpha$ -carbon

atom or present as an N-substituent on the amine group of the N-substituted glycine. Thus, in this interesting embodiment of the invention  $R^1$  and  $n$ , when used in connection with formula IIb above, are preferably the same as discussed in connection with formula IIa and IIc above, i.e.  $n$  is preferably 1 or 2, in particular 1 and  $R^1$  is preferably selected from the group consisting of cyclopentyl, cyclohexyl and cycloheptyl, in particular cyclohexyl.

As it appears from the examples provided herein, in the  $X^3$  and  $X^8$  positions, amino acids having a hydrophobic side chain or a hydrophobic N-substituent seem to enhance the binding properties of the peptide to the receptor, which could be due to hydrophobic interactions between the side chains or N-substituents of the  $X^3$  and  $X^8$  amino acids and hydrophobic residues in the uPAR binding region. Thus,  $X^3$  and  $X^8$  are preferably selected from amino acids with side chains or N-substituents with a hydrophobicity constant ( $\pi$ ) of at least 0.5.

The hydrophobicity constant ( $\pi$ ) expresses the hydrophobicity of the side chain or N-substituent relative to the hydrogen atom (or, in other words: The hydrophobicity constant ( $\pi$ ) expresses the hydrophobicity of the acetylated amino acid amide in question relative to acetylglycine amide).

In the present context, the hydrophobicity constant ( $\pi$ ) is defined as described in J.L. Fauchère et al. (1988) *Int. J. Peptide Protein Res.* 32:269-278:

$$\pi = \log P_{(\text{Ac-amino acid-NH}_2)} - \log P_{(\text{Ac-Gly-NH}_2)} \quad (\text{Q})$$

wherein  $P$  is the partition coefficient of the acetylated amino acid amide in the water-octanol system.

Expression Q is an application of the Hansch equation (S) (C. Hansch et al. (1964), *J. Am. Chem. Soc.* 86: 5175, Hansch et al. (1971) *Chem Rev.* 71: 525) to amino acids:

$$\pi = (\log P) / (\log P_0) \quad (\text{S})$$

wherein  $P$  is the partition coefficient of the substituted compound and  $P_0$  is the partition coefficient of the parent compound in the water-octanol system.

Thus, the hydrophobicity of any given amino acid may readily be expressed by the above-defined  $\pi$ -value.  $\pi$ -values for a number of amino acid side chains can be found in e.g. J.L. Fauchère et al. (1988) *Int. J. Peptide Protein Res.* 32:269-278, or, as an alternative, the  $\pi$ -value of the amino acid in question can be calculated in accordance with formula Q defined  
5 above, using the water-octanol partition constant (P) for the corresponding acetylated amino acid amide. The water-octanol partition constant (P) may be determined by methods known in the art, e.g. such as described in V. Pliska et al. (1981) *J. Chromatogr.* 216:79-92.

10 As discussed above, formula Q typically relates to the hydrophobicity constant ( $\pi$ ) of the side chain of an amino acid relative to hydrogen. However, since formula Q is merely an extension of the Hansch equation (S) specifically to amino acids, it is easily applicable to N-substituted amino acids given that in this circumstance, the term also relates to the hydrophobicity of a group relative to that of hydrogen. Thus, the partition coefficient of N-  
15 substituted glycines can be compared to that of glycine (once derivatised to their corresponding acetylated glycine amides), as can the partition coefficient of any N-substituted amino acid since the original Hansch equation (S) relates to the *additive* contribution to the hydrophobicity constant by a substituent relative to that of the hydrogen-containing parent compound

20

As it appears from the formula Q, defined above, the hydrophobicity constant ( $\pi$ ) of glycine is zero as the  $\pi$ -value of the amino acid side chain is measured relative to the hydrogen atom.

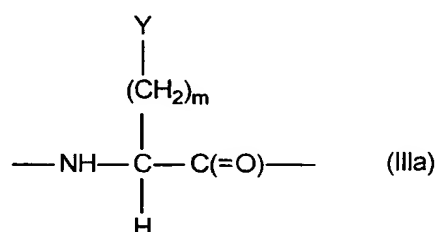
25 In preferred embodiments of the invention, the side chains or N-substituents of the  $X^3$  and  $X^8$  amino acids have a hydrophobicity constant ( $\pi$ ), as defined above, of at least 0.75, preferably at least 1.0, such as at least 1.5, e.g. at least 2.0, or even as high as at least 2.5.

Specific examples of suitable amino acids to be incorporated in the  $X^3$  and  $X^8$  positions are  
30 e.g. aromatic L- and D-amino acids, such as D- and L-phenylalanine, D- and L-tryptophan, D- and L-tyrosine, D- and L-histidine,  $\beta$ -2-naphthyl-L-alanine,  $\beta$ -2-naphthyl-D-alanine,  $\beta$ -1-naphthyl-L-alanine,  $\beta$ -1-naphthyl-D-alanine as well as aromatic N-substituted glycines, such as N-(2,3-dimethoxybenzyl)glycine, N-(3-indolyethyl)glycine, N-benzylglycine, N-(methylnaphthalyl)glycine, N-(2,2-diphenylethyl)glycine, N-(indanyl)glycine, N-(2-ethyl-2-  
35 pyridinyl)glycine, N-(4-methoxyphenylethyl)glycine. Examples of amino acids which are

especially suitable for incorporation in the X<sup>3</sup> and X<sup>8</sup> position, respectively, are L-phenylalanine in the X<sup>3</sup> position, and L-tryptophan, N-benzylglycine, N-(methylnaphthalyl)glycine or N-(2,3-dimethoxybenzyl)glycine in the X<sup>8</sup> position.

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In an interesting embodiment of the invention the amino acid X<sup>4</sup> is selected from L- and D-amino acids of the general formula IIIa



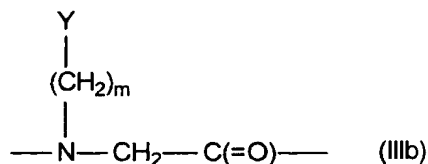
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wherein m is an integer in the range from 1 to 3, such as 1, 2 or 3, and Y is a group capable of donating and/or accepting one or more hydrogen bonds such as OH, SH, NH<sub>2</sub>, CONH<sub>2</sub>, COOH and OPO<sub>3</sub>H.

15

Specific amino acids of the general formula IIIa which are considered to be especially suitable for incorporation in the X<sup>4</sup> position are L-serine, D-serine, L-cysteine and D-cysteine, in particular D-serine.

20 Furthermore, it is envisaged that N-substituted glycines may also be suitable as the X<sup>4</sup> amino acid. Thus, in another interesting embodiment of the invention X<sup>4</sup> is selected from N-substituted amino acids of the below formula IIIb:

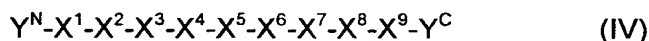


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wherein m and Y are as defined above, i.e. m is preferably 1 or 2, in particular 1, and Y is group capable of donating and/or accepting one or more hydrogen bonds such as OH, SH, NH<sub>2</sub>, CONH<sub>2</sub>, COOH and OPO<sub>3</sub>H, in particular OH and SH. Thus specific examples of N-substituted amino acids which are envisaged to be suitable X<sup>4</sup>-amino acids are, for  
 5 example, N-(hydroxymethyl)glycine, N-(hydroxyethyl)glycine, N-(methylthiol)glycine and N-(ethylthiol)glycine.

The peptide of the invention, comprising the amino acid sequence as defined in formula I above, may of course consist of the peptide fragment X<sup>1</sup>...X<sup>9</sup> exclusively, or the peptide  
 10 may consist of two or more repeating units of the peptide fragment X<sup>1</sup>...X<sup>9</sup>.

In another interesting embodiment of the invention, the peptide has the general formula IV



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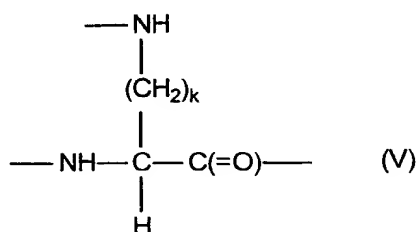
wherein Y<sup>N</sup> is an N-terminal group, Y<sup>C</sup> is a C-terminal group, and X<sup>1</sup>...X<sup>9</sup> is the peptide fragment I as defined above.

The N-terminal group (Y<sup>N</sup>) may be such groups which are readily attached to an N-terminal  
 20 amino acid of a peptide such as C<sub>1-4</sub>-alkyl, C<sub>1-4</sub>-alkylcarbonyl such as acetyl, optionally substituted arylcarbonyl, and optionally substituted heteroarylcarbonyl. The N-terminal group, however, may also be hydrogen (i.e. the N-terminal of the peptide is the free amine of the first amino acid (X<sup>1</sup>) of the peptide fragment) or a peptide radical having 1-7 amino acids.

25

In a similar way, the C-terminal group (Y<sup>C</sup>) may be such groups which are readily attached to a C-terminal group of a peptide such as C<sub>1-4</sub>-alkoxy, C<sub>1-4</sub>-alkylamino, optionally substituted arylamino, and optionally substituted heteroarylamino. The C-terminal group (Y<sup>C</sup>), however, may also be OH (i.e. the C-terminal of the peptide is the free carboxylic acid  
 30 of the last amino acid (X<sup>9</sup>) of the peptide fragment), NH<sub>2</sub> (i.e. the C-terminal of the peptide is the amide of the last amino acid (X<sup>9</sup>) of the peptide fragment) or a peptide radical having 1-7 amino acids. In the latter case, the amino acids may be the amino acids defined above as well as protected variants thereof.

- Another possible meaning for the group  $Y^C$  is a -linker- (peptide having 1-7 amino acids) moiety, which may be used with the aim of protecting a peptide according to the present invention against proteolytic degradation. This principle where a pre-sequence is attached to the peptide via a linker, e.g. an  $\alpha$ -hydroxy carboxylic acid linker, has been suggested by
- 5 Holm and Larsen in WO 98/11126. Thus,  $Y^C$  may specifically be a radical  $Y'^C$  with the formula  $-O-CH(R)-C(=O)-$ (peptide having 1-7 amino acids) where R is a radical as defined in WO 98/11126. The preferred embodiments described in WO 98/11126 also applies in this case.
- 10 In one interesting embodiment of the invention the total molecular weight of a peptide of the general formula IV is below about 3,000 g/mol, irrespective of the actual N- and/or C-terminal groups employed.
- In another interesting embodiment, the peptide of the invention comprises more than one
- 15 peptide fragment as defined in formula I above and as will be understood from the examples provided herein, a very promising approach for incorporation of two or more peptide fragments, as defined above, is to attach each of the peptide fragments to a common scaffold.
- 20 In principle, various polyfunctional molecules, such as di-, tri-, tetra-, and pentafunctional molecules, which are capable of reacting with the N-terminal and/or the C-terminal of a peptide fragment as defined above can be used as the scaffold to which the peptide fragments are attached. However, in a preferred embodiment of the invention, the common scaffold is an L- or D-diamino acid of the general formula V



25

wherein k is an integer in the range from 1 to 6, preferably in the range from 2 to 5, such as 3 or 4.

Preferred examples of amino acids of the general formula V is L-lysine, D-lysine, L-  
 30 ornithine and D-ornithine, in particular L-lysine.



Thus, it should be understood that the C-terminal amino acid of a first peptide fragment (i.e. the X<sup>9</sup> amino acid) is coupled to the  $\alpha$ -amino group of the scaffold and that the C-terminal amino acid of a second fragment is coupled to the  $\omega$ -amino group of the scaffold with the  
5 general formula V. However, for symmetry reasons it may be advantageous to incorporate one or more linkers between the C-terminal of the peptide fragment and the scaffold to which the peptide fragment is attached.

Thus, in an interesting embodiment of the invention one or more of the peptide fragments  
10 are linked to a scaffold of the general formula V as defined above, via one or more  $\omega$ -amino acids linkers of the general formula VI



wherein j is an integer in the range from 1 to 6, preferably in the range from 1 to 4, such as  
15 in the range from 1 to 3, e.g. 1, 2 or 3. Specific examples of suitable  $\omega$ -amino acids of the general formula VI are e.g. glycine,  $\beta$ -alanine and  $\gamma$ -amino butyric acid, in particular glycine and  $\beta$ -alanine.

This being said, it will be understood by the skilled person that several other possibilities  
20 exist for linking two or more peptide fragments of the general formula I to a common scaffold. The peptide fragments may for example be incorporated in, or conjugated with, a molecular carrier such as a protein, e.g. human albumin and other mammalian albumins; diphtheria toxin, ricin toxin and human enzymes for activation for of prodrugs, e.g. recombinant human betaglucuronidase; interleukins and other cytokines which activates  
25 cell-mediated attack on tumour cells; human immunoglobulins, e.g. IgG; human protease inhibitors, such as alpha-2-antiplasmin, alpha-1-antitrypsin, inter-alpha-trypsin inhibitor, bikunin, Kunitz-type protease inhibitors, kallistatin, secretory leukoprotease inhibitor (SLPI), elafin, or tissue inhibitors of metalloproteases such as TIMP-1, TIMP-2, TIMP-3 and TIMP-4. Other interesting examples of scaffolds are polypeptides, e.g. polylysines, polyhistidines  
30 or polyornithines as well as natural or synthetic polymeric molecules such as natural and synthetic polysaccharides and derivatives thereof, for example dextrans and dextran-derivatives, starches and starch derivatives, cellulose derivatives, amylose and pectin or synthetic polymers having functional groups such as polyvinyl alcohols, polyallyl alcohol, polyethylene glycols and substituted polyacrylates

It is important, of course, that the molecular carrier (i.e. the common scaffold) possesses at least two, but preferably more, functional groups capable of reacting with either the C-terminal group and/or the N-terminal group of the peptide fragment. Very interesting  
5 scaffolds of the invention are therefore such scaffolds which have a dendritic structure carrying a plurality of amino, carboxy, hydroxy and/or thiol groups.

Recently fusion proteins or chemical conjugates have been constructed which contain a uPAR ligand sequence together with a partner protein that sterically hinders accessibility of  
10 neighbouring cell-surface receptors and prolongs the half-life of the competitor in the blood circulation. The range of suitable fusion or conjugate protein partners is of course limitless, and includes already uPA or plasmin inhibitors, toxins and antibodies. However, as well as enabling interference with the cell-surface proteolytic system of tumour cells, this range of partner proteins for antagonists can be extended to facilitate the specific targeting of uPAR-  
15 expressing cells. For example this could employ enzymes which process prodrugs (using e.g. ADEPT: K. Bagshawe (1995) *Drug. Develop. Res.* 34:220-230) or antibodies directing cell-mediated cytotoxic reactions (H. Wolf et al. (1994) *Rec. Results Cancer Res.* 135:185-195) or even packaged delivery systems (e.g. virosomes: R. Bron et al. (1994) *Biochemistry* 33:9110-9117) may exploit many different methods of attack on tumour cells  
20 which display uPAR on their surface. Thus, following peptide synthesis, the non-coding amino acid peptide sequence of the general formula I can be chemically conjugated to any of the above mentioned scaffolds. Examples of methodologies for the preparation of fusion proteins (recombinant joining together of two protein sequences) are given in Min et al., *Cancer Research* 56:2428-2433 (1996) and in Kobayachi et al., *J. Biol. Chem.* 270:8361-  
25 8366 (1995).

Specific examples of polymeric carrier molecules which may act as a "common scaffold" are e.g. human serum albumin, human lysozyme (a naturally occurring low molecular weight basic protein), polylysine comprising from 3 to 10 free lysine residues, etc. An  
30 important issue in connection with the selection of polymeric carrier is the antigenicity, which will be a limiting factor, as will be known for the person skilled in the art.

It should be understood that the peptides of the invention may also be in the form of a salt thereof, of which pharmaceutically acceptable salts are especially relevant. Salts include  
35 acid addition salts and basic salts. Examples hereof are hydrochloride salts, hydrobromide

salts, sodium salts, calcium salts, potassium salts, etc. Pharmaceutically acceptable salts are, e.g., those described in "Remington's Pharmaceutical Sciences", 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions, and in Encyclopedia of Pharmaceutical Technology. Furthermore, the peptides of  
5 the invention may also be present in a hydrate form.

The peptides of the invention may be prepared by methods known per se in the art. Thus, the peptides of the invention may be prepared by standard peptide-preparation techniques such as solution synthesis or Merrifield-type solid phase synthesis using well-known  
10 standard protection, coupling and deprotection procedures. Thus, the peptides of the invention may be produced by a solid phase method essentially comprising the following steps:

- a) coupling an N- $\alpha$ -protected amino acid to a solid support material, thereby forming an  
15 immobilised N- $\alpha$ -protected amino acid,
  - b) removing the N- $\alpha$ -protecting group, thereby forming an immobilised amino acid having an unprotected N-terminal end,
  - 20 c) coupling an additional N- $\alpha$ -protected amino acid in the carboxyl activated form to the N-terminal end of the immobilised amino acid fragment, and
- repeating the removal/coupling step procedure in step b) and c) until the desired peptide is obtained, and then
- 25 d) cleaving off the peptide from the solid support material.

The coupling, removal and cleavage step is performed by methods known to the person skilled in the art taking into consideration the protection strategy and the selected solid  
30 phase material. In general, however, it is believed that the Boc (tert.butyloxycarbonyl) as well as the Fmoc (9-fluorenylmethyloxycarbonyl) protection strategies are applicable and that peptide bonds may be formed using the various activation procedures known to the person skilled in the art, e.g. by reacting a C-terminal activated derivative (acid halide, acid anhydride, activated ester e.g. HOBt/carbodiimide-ester, etc.) of the appropriate amino acid

with the amino group of the relevant amino acid or peptide as known to a person skilled in peptide chemistry.

- Furthermore, it may be necessary or desirable to include side-chain protection groups
- 5 when using amino acid units carrying functional groups which are reactive under the prevailing conditions. The necessary protection scheme will be known to the person skilled in the art (see e.g. M. Bodanszky and A. Bodanszky, "The Practice of Peptide Synthesis", 2. Ed, Springer-Verlag, 1994, J. Jones, "The Chemical Synthesis of Peptides", Clarendon Press, 1991, and A. Dryland and R.C. Sheppard (1986) J. Chem. Soc., Perkin Trans. 1,
- 10 125-137). Examples of suitable solid support materials for use in the solid phase synthesis of the peptides are e.g. functionalised resins such as polystyrene, polyacrylamide, polydimethylacrylamide, polyethyleneglycol, cellulose, polyethylene, polyethyleneglycol grafted on polystyrene, latex, dynabeads, etc.
- 15 In addition, it may be necessary or desirable that the C-terminal amino acid of the peptide is attached to the solid support material by means of a common linker such as 2,4-dimethoxy-4'-hydroxy-benzophenone, 4-(4-hydroxy-methyl-3-methoxyphenoxy)-butyric acid, 4-hydroxy-methylbenzoic acid, 4-hydroxymethyl-phenoxyacetic acid, 3-(4-hydroxymethylphenoxy)propionic acid, and p-[(R,S)-a[1-(9H-fluoren-9-yl)methoxy-
- 20 formamido]-2,4-dimethoxybenzyl]-phenoxy-acetic acid.

- In cases where N-substituted amino acids (such as N-substituted glycines) are incorporated into the fragment of the peptide of the invention, the peptides of the invention may in part be prepared by methods described for the preparation of peptoids to provide
- 25 peptide-peptoid hybrids known as peptomers (Østergaard, S. and Holm, A. (1997, *Peptomers: a versatile approach for the preparation of diverse combinatorial peptidomimetic bead libraries*. Mol. Divers. 1997, Dec, 3(1):17-27; and Zuckermann, R. N., Kerr, J. M., Kent, S. B. H. and Moss, W. H. (1992) *Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid-phase synthesis*. J. Am.
- 30 Chem. Soc. 114, 10646-10647). N-substituted glycines can be inserted at any point of the growing peptide (or peptomer) chain. The new linkage may be termed a peptoid and comparison of their backbones reveals that the side chain functionality of a peptoid moiety is attached to the amide nitrogen as opposed to the  $\alpha$ -carbon in the standard peptide backbone.

One way to assemble the peptoid moiety to a pre-existing chain is from the two sub-monomers, bromoacetic acid and an amine. The coupling of bromoacetic acid to the amino terminal of a pre-existing peptide or peptomer, or to an amino group on the polymer-support resin, using a carbodiimide, is followed by addition of a primary amine. This  
5 sequence of reactions can be repeated as desired. Another way to assemble the peptoid is from the C-terminal end of the peptide or peptomer by reversing the coupling order. To the C-terminal portion of a pre-existing peptide or peptomer is coupled a primary amine, using a carbodiimide, to give a peptoid linkage to which is added bromoacetic acid. This sequence of reactions regenerates a carboxylic acid amenable to further peptide or peptoid  
10 assembly.

Among the real advantages of peptoids (i.e. peptides comprising N-substituted amino acids, such as N-substituted glycines) relative to other unnatural peptide-like polymers is their ease of synthesis, the availability of a wide range of monomers (essentially, all  
15 primary amines are suitable units for peptoid synthesis), and their high stability towards proteases. Moreover, peptoids allow for a refinement in the shape of the peptide by effectively allowing a side chain to be transposed from the  $\alpha$ -carbon of an amino acid to its amine. Given the number of amino acids and primary amines amenable to this methodology and their putative combinations within a peptomer chain, the extent of the  
20 peptomer library is immeasurable giving diverse peptidomimetic bead libraries.

The peptides of the invention may be cleaved from the solid support material by means of an acid such as trifluoroacetic acid, trifluoromethanesulfonic acid, hydrogenbromide, hydrogenchloride, hydrogenfluoride, etc. optionally in combination with one or more  
25 "scavengers" suitable for the purpose, e.g. ethanedithiol, triisopropylsilan, phenol, thioanisole, etc., or the peptides of the invention may be cleaved from the solid support by means of a base such as ammonia, hydrazine, an alkoxide, such as sodium ethoxide, an hydroxide, such as sodium hydroxide, etc.

30 Another possible strategy is to separately prepare two or more of the sequences constituting the peptide of the invention by solution synthesis, solid phase synthesis, recombinant techniques, or enzymatic synthesis, followed by coupling of the two (or more) sequences by well-known segment condensation procedures, either in solution or using solid phase techniques or a combination thereof.

In the research carried out in connection with the present invention a large number of peptides have been screened for the desired activity (i.e. inhibition of the uPA/uPAR interaction). As will be understood from the examples provided herein the initially screened peptides were synthesised using the so-called "bead library technology" approach by Lam et al. Nature 354:82-84 (1991) and the principle of the method is described in Fig. 1.

The method is initiated by the distribution of all the resin beads in portions of equal size. An amino acid (monomer) is attached (coupled) to each portion but only one type of monomer to each portion. After coupling has completed the portions are combined and mixed thoroughly before the next dividing step. Fig. 1 illustrates the synthesis by using only 3 building blocks and two steps, but the number of building blocks and synthesis steps is of course optional.

The procedure generates a very large library of compounds (peptides) in a random fashion. The important thing is that each bead only represents one sequence, i.e. an amount of e.g. 2 million beads will generate 2 million different peptides.

The screening procedure is accomplished by methods known in the art, i.e. the library is typically incubated with a water soluble receptor of interest (target receptor). Usually alkaline phosphatase is coupled either directly or indirectly to this receptor. This enzyme can be attached to the target receptor by the binding of a phosphatase conjugated antibody directed against the target receptor, or alkaline phosphatase may be conjugated to streptavidin which is then directed against a biotinylated form of the target receptor or a biotinylated anti-receptor antibody. By using conventional immunochemical techniques, in which the enzyme catalyses a reaction which generates a colour, active beads that bind to the target receptor can be made visible to the eye. Once the active beads are found they are isolated and the sequence is analysed one bead at a time using a protein sequencer. Following this, the peptide or peptides of interest may be synthesised in full-scale as described above.

30

Although the peptides of the invention in general exhibit a high affinity for uPAR, low off-rates and high potency in a cell-binding system there may, however, be a variation between the potency of the individual peptides described herein. Therefore, the present inventors have provided suitable assays enabling the person skilled in the art to select

effective and preferred peptides based on such assays. Examples of these assays are given in the experimental section of the present description.

In the present context, the term "a receptor binding form of uPA" is intended to mean any  
5 form of uPA possessing a site that binds to a site at a uPAR, that is to say that the uPA contains the uPAR binding site. The receptor binding form of uPA can thus be pro-uPA, uPA, an amino-terminal fragment of uPA (ATF-uPA), a uPA that is irreversibly inhibited by e.g. diisopropyl fluorophosphate (DFP), p-nitrophenyl-p'-guanidinobenzoate (NPGB), or any other modification of uPA that can bind to a uPAR.

10

The usage of the term "a uPAR" indicates that even though the polypeptide part of uPAR in a species might be the same for all uPARs, there is a plurality of uPARs as for example the carbohydrate part or the mechanism of surface attachment of the uPAR can be different. It may even be that some cells, e.g. cancer cells, have substantially different uPARs which  
15 might have important therapeutic significance as it might be possible to block the binding of uPA to uPARs residing on a cancer cell without affecting the binding of uPA to uPARs on non-pathological cells or of specifically killing cancer cells that express uPAR.

The enzyme urokinase-type plasminogen activator (uPA) has only one well-defined  
20 macromolecular substrate, namely plasminogen. By cleavage at Arg<sup>560</sup>, plasminogen is activated to the broad spectrum protease plasmin. By the term "inhibiting the activation of plasminogen to plasmin by inhibiting the binding of a receptor-binding form of uPA to a uPAR" is therefore meant that this activation by uPA is substantially inhibited or a situation where the activation is sufficiently inhibited so as to inhibit or reduce the undesired effect of  
25 the plasmin, i.e. the proteolytic activity of plasmin is prevented or counteracted.

The prevention of the binding of a receptor binding form of uPA to a uPAR is, e.g. suitably performed by blocking the uPAR by administration, to the mammal, of a substance binding to the uPAR so as to occupy a site of the receptor to which a receptor binding form of uPA  
30 is normally bound, the substance being administered in an amount effective to reduce the binding of the receptor binding form of uPA to the receptor. In the present context the term "blocking the uPAR" means that a substance that is not able to activate plasminogen to plasmin is bound to uPAR, preferably by a substantially irreversible binding, thereby preventing a receptor binding form of uPA from catalyzing the conversion of cell surface bound  
35 plasminogen into plasmin.

The requirements of the peptides of the invention to function as efficient inhibitors of the uPA/uPAR system is that on the one hand the peptide should have a high affinity for the receptor, and on the other hand that the peptide should "stay" on the receptor as long as possible. Thus the peptide/uPAR system should preferably exhibit slow dissociation kinetics (the so-called "off-rate"), which may conveniently be expressed as the dissociation rate constant  $k_{\text{diss}}$ .

Therefore, assays have been developed which give an assessment of the capability of the peptide/uPAR system to exert the desired affinity and/or kinetic properties, i.e. to assess the capability of the peptides of the invention to function as inhibitors of the uPA/uPAR system and thereby impede efficient binding of uPA to the receptor.

Based thereon, peptides of the invention which are especially preferred are peptides which, when tested in the Biacore Binding Assay described herein, have a dissociation rate constant ( $k_{\text{diss}}$ ) relative to the Growth Factor Domain of uPA (GFD) of at the most 5.0, such as at the most 4.0, e.g. at the most 3.0, preferably at the most 2.0, such as at the most 1.5, e.g. at the most 1.0, most preferably at the most 0.75, such as at the most 0.5, e.g. at the most 0.25, at the most 0.1, at the most 0.05, or at the most 0.01.

On the other hand, the uPA/uPAR Binding Assay disclosed in the examples, is a test for determining the thermodynamic binding properties of a given peptide to the receptor in a cell-binding system. Thus, very interesting peptides of the invention are such peptides which when tested in the uPA/uPAR Binding Assay described herein, are able to inhibit the binding of radio-labelled Amino Terminal Fragment (ATF) of uPA to cell-surface uPAR by 50% at a concentration of at the most 50 nM, such as of at the most 35 nM, e.g. of at the most 25 nM, preferably of at the most 10 nM, such as of at the most 7.5 nM, e.g. of at the most 5 nM, most preferably of at the most 3 nM, such as of at the most 2 nM, e.g. of at the most 1 nM, in particular of at the most 0.5 nM, such as of at the most 0.1 nM, e.g. of at the most 0.01 nM.

Evidently, it is preferred that a peptide of the invention fulfils one or preferably both of the above criteria on at least the stated lowest level, more preferably on the intermediate level and most preferably at the stated highest level.



Based on experiments of the above-mentioned type carried out so far, examples of especially interesting peptides are such peptides, wherein the at least one peptide fragment is selected from the group consisting of dChaFsrYLWS, SLChaFsQYLWS, eChaFsyYLWS, DChaFsrYLWS, DChaFsrYLWS, dChaFsrYLWS, tChaFsrYLWS, 5 dChaFsrYL<sup>2</sup>nAS, DChaFsRYLWS, DChaFsrYL<sup>1</sup>nAS, eChaFsYYLWS, D-Cha-F-s-r-L-L-W-h, D-Cha-F-s-r-Cha-L-W-I, D-Cha-F-s-r-Y-L-Nal-h, D-Cha-F-s-r-DMB-f-TRA-MEA, D-Cha-F-s-r-DMB-f-Bzl-MEA, D-Cha-F-s-r-DMB-f-AMN-MEA and D-Cha-F-s-r-DMB-f-DMB-I

wherein Cha designates  $\beta$ -cyclohexyl-L-alanine, <sup>1</sup>nA designates  $\beta$ -1-naphthyl-L-alanine, 10 <sup>2</sup>nA designates  $\beta$ -2-naphthyl-L-alanine, capital letters designate L-amino acids, lower case letters designate D-amino acids,  $\beta$ A designates  $\beta$ -alanine, DMB designates N-(2,3-dimethoxybenzyl)glycine, TRA designates N-(3-indolyethyl)glycine, MEA designates N-(2-methoxyethyl)glycine, Bzl designates N-benzylglycine and AMN designates N-(methylnaphthalyl)glycine.

15

Other examples of specific peptides which fulfil the requirements set forth above are peptides selected from the group consisting of  $\alpha$ -[DChaFsrYLWSG]- $\epsilon$ -[DChaFsrYLWSG]-(L)-lysine,  $\alpha$ -[DChaFsrYLWSG $\beta$ A]- $\epsilon$ -[DChaFsrYLWSG]-(L)-lysine, and  $\alpha$ -[DChaFsrYLWS $\beta$ A]- $\epsilon$ -[DChaFsrYLWS]-lysine.

20

where  $\beta$ A designates  $\beta$ -alanine, and Cha, capital letters and lower case letters are as defined above.

The present invention also relates to a test system comprising methods for selecting a 25 peptide antagonist which is suitable for preventing or counteracting localized extracellular proteolytic activity of plasmin in a human. The inhibition of the proteolytic activity of plasmin is performed by inhibiting the activation of plasminogen to plasmin by inhibiting the binding of a receptor-binding form of uPA to a uPAR in the human.

30 The method comprises providing a modified uPAR of a non-human mammalian species, said modified uPAR being modified in a manner which renders it capable of being antagonized by a peptide antagonist while retaining its capability of binding to a receptor-binding form of uPA of said mammal species substantially unchanged, in a model system for assessing antagonism of uPA/uPAR binding and comprising said modified uPAR carried by 35 cells of the non-human mammal species as well as a receptor-binding form of uPA of the

species. The method will be performed by subjecting a panel of peptides to assessment in the model system and selecting, as peptide antagonists, such peptides among the panel of peptides which, in the model system, result in a degree of antagonism of the binding of the uPA to the modified uPAR which is similar to the degree of antagonism obtained by using an ATF-fragment of said uPA. The degree of antagonism is preferably at least one-tenth of the degree of antagonism obtained by using an ATF-fragment, more preferably at least one-fifth of the degree of antagonism obtained by using an ATF-fragment, more preferably at least one-quarter of the degree of antagonism obtained by using an ATF-fragment, even more preferably half of the degree of antagonism obtained by using an ATF-fragment, preferably three-quarters of the degree of antagonism obtained by using an ATF-fragment, most preferably equal to or higher than the degree of antagonism obtained by using an ATF-fragment.

The non-human species of the screening method may be selected from the group consisting of mouse, hamster, rat, dog, cat, cow, rabbit or non-human primates or any other mammal.

Furthermore, the model system can comprise human, non-human or non-human primate tumour cells in an environment of the non-human cells. The modified uPAR may be a uPAR the natural counterpart of which is associated with the particular tumour cells or the environment of the non-human cells or both. Also the receptor-binding form of uPA may be supplied by either the particular tumour cells or the environment of the non-human cells.

The described model system can be comprised of one or more of the following test systems:

- 1) a screening assay in which the possible inhibition of uPA/uPAR interaction by antagonist is determined by adding the antagonist to the system comprising the modified uPAR and solubilized uPA, uPA bound to uPAR being detected by being labelled or by means of a labelled anti-uPA antibody, or adding the substance to a system comprising immobilized uPA and solubilized uPAR, uPAR bound to uPA being detected by being labelled or by means of a labelled anti-uPAR antibody,
- 2) an assay in which the possible inhibition of uPA/uPAR interaction by the antagonist is determined by adding the antagonist to a system comprising uPAR and ra-

diolabelled uPA or a derivative thereof, cross-linking any uPAR bound to uPA and detecting any cross-linked product by SDS PAGE and autoradiography,

- 3) an assay in which the possible inhibition of binding of uPA to uPAR on the surface  
5 of cultured cells is determined by adding the antagonist to a system comprising radiolabelled uPA or a derivative thereof and cells carrying uPAR and detecting any uPA or derivative binding to uPAR by gamma counting of the cells,
- 4) an assay in which the possible inhibition of cell surface plasminogen activation by  
10 receptor-bound exogenous pro-uPA is determined by adding the antagonist to cells carrying uPAR and subsequently adding pro-uPA, followed by measurement of plasmin generation on the cell surface,
- 5) an assay in which the possible inhibition of cell surface plasminogen activation by  
15 receptor-bound endogenous pro-uPA is determined by incubating cells carrying uPAR and producing pro-uPA with the antagonist, followed by measurement of plasmin generation on the cell surface,
- 6) an assay in which human or non-human tumour cells are inoculated into an immu-  
20 nocompromised mouse and recovered from the mouse after treatment with the antagonist, followed by measurement of the reduction of bound uPA on the tumour cell surface using flow cytometric methods,
- 7) administering a substance which has been established to inhibit uPA/uPAR inter-  
25 action to a immunodeficient mouse which also lacks a functional gene for uPAR, said mouse being susceptible to invasion or metastasis by human or non-human tumour cells and said mouse being inoculated with human or non-human tumour cells which are known to invade and/or metastasize in the presence of uPA and uPAR and which are capable of invasion and/or metastasis in the mouse, and  
30 measuring the inhibition of the invasion and/or metastasis of the human or non-human tumour cells in the mouse.

In 6) of the above test systems the amount of uPA bound to uPAR on the surface of the tumour cells is then determined by either 1) acid elution and immunocapture assay (see

e.g. Tapiovaara et al, 1993, Blood 82, 914-919) or 2) flow cytometry with anti-uPA antibody fluorescent staining.

In 7) of the above test systems the tumour cells inoculated into the mouse may, prior to  
5 their inoculation, have been transduced with the lacZ gene which encodes the enzyme  $\beta$ -D-galactosidase. This enzyme will give rise to a blue staining when subjected to the substrate X-gal. Thus, this system makes it possible to obtain a distinct colour difference between the human cancer cells and the mouse's own cells, thereby very considerably facilitating detection and quantitation of invading cells and metastases.

10

The modified uPAR of the screening method is a non-human uPAR in which the amino acid sequence has been modified by substituting one or more amino acids of the non-human uPAR. The modification is obtained by substituting 10 amino acids, preferably 5 amino acids, more preferably 3 amino acids, even more preferably 2 amino acids, most preferably  
15 1 amino acid. The region wherein the substitution of the amino acid(s) is/are preferred is located in uPAR domain III loop 3 (see figure 9). The preferred amino acid residue(s) to be substituted is/are the amino acid(s) of the non-human uPAR corresponding to the amino acid residues His<sup>249</sup>, Ser<sup>254</sup> and Asn<sup>256</sup> of the human uPAR sequence, most preferably the amino acid of the non-human uPAR to be substituted is the amino acid corresponding to  
20 amino acid His<sup>249</sup> of the human uPAR sequence (marked with an arrow in figure 9). The substitution in the amino acid sequence of the non-human uPAR is preferably so that the amino acid at the substituted position in the non-human uPAR is the same as the amino acid of the human uPAR, i.e. the amino acid marked with an arrow in figure 9 of the non-human uPAR sequences is a His residue, e.g. Gly<sup>272</sup> in the mouse uPAR sequence (NCBI  
25 number sp/P35456) is substituted with His<sup>272</sup>, Gly<sup>273</sup> in the rat uPAR sequence (NCBI numbers gi/2253496, pir//S42152, and sp/P49616) is substituted with His<sup>273</sup>, Ser<sup>268</sup> in the bovine uPAR sequence (NCBI number sp/Q05588) is substituted with His<sup>268</sup>. In the hamster the Gly<sup>251</sup> (according to the sequence alignment in the published hamster uPAR sequence (Fowler et al, Thromb. Haem. 1998, 80,148-154)) is substituted with His<sup>251</sup>. The  
30 non-human sequences corresponding to the critical histidine in the human uPAR sequence are also obvious from the alignment diagram published in Fowler et al, Thromb. Haem. 1998, 80,148-154.

The method by which this amino acid substitution is obtained could be any method known  
35 in the art for substituting amino acid sequences in proteins, e.g. gene knock-in/gene knock-

out technology (Hanks et al 1995, Science 269, 679-682). In the case of using a mouse as the non-human mammal of the screening method, the mouse may be a transgenic mouse containing the modified uPAR.

- 5 The present invention also relates to a pharmaceutical composition comprising a peptide as defined above. Such pharmaceutical compositions may be in a form (e.g. in liquid, powdered or lyophilised form) adapted to oral, parenteral (intravenous, intraperitoneal, intramuscular, subcutaneous) rectal, intranasal, dermal, vaginal, buccal, ocularly or pulmonary administration, and such compositions may be prepared in a manner well-
- 10 known to the person skilled in the art, e.g. as generally described in "Remington's Pharmaceutical Sciences", 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions and in the monographs in the "Drugs and the Pharmaceutical Sciences" series, Marcel Dekker.
- 15 Thus, the peptides used in this invention may be prepared as formulations in pharmaceutically acceptable media, for example, saline, phosphate buffered saline (PBS), Ringer's solution, dextrose/saline, Hank's solution, and glucose. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting
- 20 agents, emulsifying agents, detergents, and the like. Additives may also include additional active ingredients, e.g. bactericidal agents, stabilizers, or preservatives. The amount administered to the patient will vary depending upon what is being administered, the purpose of the administration, such as prophylaxis or therapy, the state of the host, the manner of administration, and the like.
- 25
- The pharmaceutical compositions are typically intended for transdermal or parenteral administration, e.g. intravenously, subcutaneously, or intramuscularly. Orally administrative forms are also desired and can be provided by modifying the composition to bypass the stomach environment. The composition can be used for prophylactic and/or therapeutic
- 30 treatment. Preferably, the pharmaceutical compositions are administered intravenously. Thus, the compositions may comprise a peptide antagonist according to the invention which is dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. Such compositions may be sterilised by conventional sterilisation techniques, or may be sterile filtered.

The resulting aqueous solutions may be packaged for use as is, or lyophilised, the lyophilised preparation being combined with a sterile aqueous carrier prior to administration. The peptide antagonist may also be administered with a second biologically active agent, such as a standard chemotherapeutic agent. Such agents include but are not  
5 limited to vincristine, daunorubicin, L-asparaginase, mitoxantrone and amsacrine.

In therapeutic applications, the pharmaceutical compositions are administered to a patient in an amount sufficient to produce the desired effect, defined as a "therapeutically effective dose". The therapeutically effective dose of a peptide antagonist according to the invention  
10 will vary according to, for example, the particular use for which the treatment is made, the manner of administration, the health and condition of the patient, and the judgement of the prescribing physician. A therapeutically effective dose can be estimated by means of the desirable plasma concentration, which is large compared to the known affinity (binding constant) of the peptide for the receptor. A level of the plasma concentration of at least  
15 about 0.3 nM would often be desirable. A plasma level of at least about 10 nM would often be required, preferably at least about 100 nM, and if possible with due regard to the toxicity, side effects, etc., at least about 1  $\mu$ M. This level should be maintained for a period of e.g. one month in order to suppress tumour growth.

20 For example, the dose for continuous infusion will typically be in the range of about 0.1 mg to about 1000 mg per day for a 70 kg patient, preferably between about 1 mg and about 100 mg. The dose will typically be between 100 ng/kg/day and 10  $\mu$ g/kg/day.

The concentration of the peptide antagonist in the pharmaceutical formulations can vary  
25 widely, i.e. from about 0.1% to about 10%, preferably between about 0.5% and about 5% (wt/vol). The concentration will usually be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 500 ml of dextrose/saline solution and 50 mg of the peptide antagonist.

30

For solid compositions, conventional non-toxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable non-toxic composition is formed by  
35 incorporating normally employed excipients, such as those carriers previously listed, and

generally 10-95% of active ingredient, that is, a peptide antagonist substance, preferably 25-75%.

- For aerosol administration, the peptide antagonist is preferably supplied in finely divided
- 5 form along with a surfactant and propellant. Typical percentages of peptide antagonists are 0.01-20% by weight, preferably 1-10%. The surfactant must, of course, be non-toxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic
- 10 polyhydric alcohol or its cyclic anhydride such as, for example, ethylene glycol, glycerol, erythritol, arbutol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or natural glycerides may be employed.
- 15 The surfactant may constitute 0.1-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquified propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquified propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above may also be
- 20 employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided polypeptide(s) and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.
- 25 To enhance the serum half-life, the peptide antagonist may be encapsulated, introduced into the lumen of liposomes, prepared as a colloid, or other conventional techniques may be employed which provide an extended lifetime of the peptides. Thus, in certain embodiments, the peptide antagonist may be encapsulated in a liposome. A variety of methods are available for preparing liposomes, as described in, e.g. US 4,235,871; US
- 30 4,501,728; US 4,837,028 and W.H. Gottlieb et al (1992) *Cytokine* 4:385-930.

As shown in Example 9 herein, the inclusion of D-amino acids in the peptides of the invention (as well as "dimer" formation) render such peptides significant less susceptible to proteolytic degradation. For example, the most promising peptides thus far are the "dimers"

35 AE118 and AE120 (The structures of AE118 and AE120 are [DChaFsrYLWSG]<sub>2</sub>-K and

$\alpha$ -[DChaFsrYLWSG $\beta$ A]- $\epsilon$ -[DChaFsrYLWSG]-L-lysine, respectively) only showing a slight reduction in its ability to inhibit ATF binding to monolayer cultures of human MDA-MB-231 breast cancer cells after incubation in 10% mouse serum for up to 24 hours at 37 °C.

- 5 The "monomer" AE78 (i.e. the so-called "Clone 20" having the sequence: AEPMPHSLNFSQYLWYT), on the other, lost the majority of its inhibitory activity after incubation with 10% mouse serum for 24 hours even though the concentration was 1000 fold (3 orders of magnitude) higher than that of AE118.
- 10 These findings open up new prospects of administering the peptide of the invention and, as mentioned above, it may even be realistic to administer a peptide of the invention orally if the peptide comprises at least one D-amino acid, e.g. at least two D-amino acids, as it is envisaged that such D-amino acid-containing peptides may survive the proteolytic barriers prevailing in e.g. the gastrointestinal environment. Further proteolytic stability is conferred
- 15 when, as mentioned above, the fragment comprises one or more N-substituted amino acids, such as one or more N-substituted glycines. The prospect of oral administration seems most realisable in instances where the fragment comprises both D-amino acids and N-substituted amino acids, such as N-substituted glycines.
- 20 Accordingly, peptides of the invention with are considered as being of particular interest, are such peptides which, when incubated for 24 hours at 37°C in 10% mouse serum and subsequently tested in the uPA/uPAR Binding Assay, as defined herein, has an IC<sub>50</sub> value relative to the corresponding non-incubated peptide (i.e. IC<sub>50, non-incubated</sub>/IC<sub>50, incubated</sub>) of at least 0.5, such as at least 0.6, e.g. at least 0.7, preferably at least 0.8, such as at least 0.9,
- 25 e.g. at least 0.95, in particular at least 0.99.

Furthermore, the present invention relates to a peptide as defined above for use as a medicament, and to the use of a peptide as defined above for the manufacture of a medicament for the treatment of cancer. It is contemplated that the peptides of the present

30 invention are effective drugs in the treatment of a variety of different cancers. However, as the most malignant and fast lethal cancers are known to over-express uPAR it is presently believed that the most promising aspects of the present invention relates to the treatment of these malignant and fast lethal cancers, such as acute myeloid leukaemia, malignant gliomas of the brain, and gastric cancer.



## BRIEF DESCRIPTION OF THE DRAWINGS

*Fig. 1: The principle of the library technology.*

The figure shows the "divide, couple and recombine" procedure. A, B and C are the building blocks used in the synthesis step. The essential feature of the technique is the unique sequence on each bead.

*Fig. 2: Binding of peptides by combinatorial chemistry to immobilized suPAR.*

The figure shows real-time binding curves (sensorgrams) for the interaction between various ligands and immobilised uPAR measured by surface plasmon resonance (Example 3). The figure shows sensorgrams recorded for 100nM GFD, 1  $\mu$ M of AE68, 1  $\mu$ M of AE78 and 1  $\mu$ M of AE105 to immobilised, neuraminidase-treated suPAR. Also shown is the sensorgram recorded for a buffer control.

*Fig. 3: Cell-binding assay.*

The figure shows the effect of various peptide antagonists in the inhibition of the binding of ATF to monolayer cultures of human MDA-MB-231 breast cancer cells (Example 7).

*Fig. 4: Cell-binding assay.*

The figure shows the effect of AE118 (1 and 10 nM, respectively as well as DFP-uPA (10 nM)) in the inhibition of the binding of ATF to monolayer cultures of human MDA-MB-231 breast cancer cells. The experiments were carried out in the presence of 0.1% bovine serum albumin, and 0.1% and 10% fetal calf serum, respectively (Example 7).

*Fig. 5: Peptide stability in mouse serum.*

Peptides were incubated with binding serum for 0 or 24 h and then tested in binding experiments with  $^{125}$ I-labelled human ATF. The figure shows results of the cell-binding experiments performed in Example 8.

*Fig. 6: Peptide stability in mouse serum.*

Peptides were incubated for 0 to 24 h with buffer solution containing 0.1% BSA or pooled mouse serum and then tested in binding experiments with  $^{125}$ I-labelled human ATF. The figure shows the results of experiments performed in Example 8.

*Fig. 7: Result of the pharmacokinetic experiment.*

The figure shows the results of the experiments performed in Example 9.

*Fig. 8: Species specificity of uPAR interaction with immobilised peptide antagonist.*

Human and mouse suPARs were allowed to interact with AE120 in Biocore flow cells. The  
5 figure shows results of experiments performed in Example 11.

*Fig. 9: Sequence comparison of the relevant amino acids subjected to alanine mutagenesis in human uPAR to the corresponding residues of hamster, mouse, rat, and bovine uPAR.*

The sequence of human uPAR domain III loop 3 shows amino acid residues Q248, H249,  
10 H251, L252, D254, F256, S257, M258, N259, H260, I261, D262, V263 and S264. The  
sequence numbers refer to the mature human uPAR protein (without signal peptide). The  
figure shows results inter-species homology of the experiments performed in Example 12.

*Fig. 10: Dissociation rate constants for the interacting between an immobilised peptide  
15 antagonist and various suPAR variants carrying single-site mutations.*

The figure shows results of experiments performed in Example 13.

## EXAMPLES

### 20 General

#### Peptide Synthesis

The chain elongation steps of the solid-phase peptide synthesis were carried out manually  
25 using polyethylene syringes as reaction vessels. Synthesis was performed on KA-resin  
with preloaded Fmoc-amino acids on the acid-labile linker. Five equivalents of Fmoc-amino  
acids activated by 1-hydroxy-7-benzotriazole (HOBt) and N,N'-diisopropylcarbodiimide  
were used in the coupling steps and were allowed to react for more than 2 h. The Fmoc  
protecting group was removed with 20% piperidine in dimethylformamide for 15-20 min.  
30 For cleavage, peptide-resins were treated for 1.5 h with 85% TFA containing 5% of phenol,  
mercaptoethanol, and thioanisole, respectively. The filtrates were concentrated by nitrogen  
flushing, and peptides were subsequently precipitated from, and washed four times with,  
diethyl ether. Peptides were finally dissolved/suspended in glacial acetic acid, lyophilised,  
redissolved in 10% acetic acid, and lyophilised again. Analytical HPLC analysis was  
35 performed on a C<sub>18</sub> column using Waters 600E equipped with Waters photodiode array

detector. A 25 min linear gradient from buffer A (0.1% TFA, 9.9% H<sub>2</sub>O, 90% CH<sub>3</sub>CN) was used. If considered necessary peptides were purified on a preparative scale. The correct identities of the peptides were confirmed by matrix assisted laser desorption ionisation mass spectroscopy or electrospray ionization mass spectrometry. The purity was checked  
5 by reverse phase HPLC.

The following peptides were synthesised using standard methodologies:

	<i>dChaFsrYLWS</i>	(Code: <i>dCha</i> )
10	<i>eChaFsyYLWS</i>	(Code: AE100)
	<i>tChaFsrYLWS</i>	(Code: AE108)
	<i>DchaFsrYLWS</i>	(Code: AE105)
	<i>DChaFsrGYLWS</i>	(Code: AE116)
	<i>DChaFsrβAYLWS</i>	(Code: AE117)
15	<i>DChaFSrYLWS</i>	(Code: AE106)
	<i>dChaFSrYLWS</i>	(Code: AE107)
	<i>SLChaFsQYLWS</i>	(Code: <i>Lcha</i> )
	<i>dChaFsrYL<sup>2</sup>nAS</i>	(Code: AE109)
	<i>DChaFsRYLWS</i>	(Code: AE110)
20	<i>DchaFsrYL<sup>1</sup>nAS</i>	(Code: AE114)
	<i>eChaFsYYLWS</i>	(Code: AE115)
	<i>SLNFSQYLWS</i>	(Code: AE68)
	<i>AEPMPSLNFSQYLWYT</i>	(Code: AE78)
	<i>arFhhYLWS</i>	(Code: AE104)
25	<i>LNFSQYLWS</i>	(Code: AE111)
	<i>DFFsrYLWS</i>	(Code: AE112)
	<i>DNFsrYLWS</i>	(Code: AE113)

The "multimeric" peptides were synthesised by standardised peptide synthesis  
30 methodologies using a orthogonal protection strategy (e.g. as described by Cwirla et al. Science, Vol 276, 1997, pp 1696-1699):

	<i>[DChaFsrYLWSG]<sub>2</sub>-K</i>	(Code: AE118)
	<i>α-[DChaFsrYLWSGβA]-ε-[DChaFsrYLWSG]-K</i>	(Code: AE120)

(Alternatively, heterogeneous methods well known to peptide chemists for construction of e.g. multimeric antigens for immunisation (D.N. Posnett et al (1988) *J. Biol. Chem.* 263:1719-1725) are also applicable within the present invention.)

## 5 Purification of proteins

The Growth factor-like domain (GFD) of human uPA was produced by endoproteinase Glu-C digestion of recombinant pro-uPA as described in M. Ploug et al. (1995) *Biochemistry* 34:12524-12534.

10

A soluble recombinant variant of human uPAR (suPAR) was expressed in Chinese hamster ovary (dhfr<sup>-</sup>) cells. The suPAR protein was purified by immunoaffinity chromatography using an anti-uPAR monoclonal antibody as described in M. Ploug et al. (1993) *Biol. Chem.* 268:17539-17546.

15

Recombinant human pro-uPA expressed in *E. coli* was provided by Dr. D. Saunders (Grünenthal, Germany). This product can be prepared as described in EP 0496327 A. Two chain active uPA was purchased from Serono (Aubonne, Switzerland).

20 The amino-terminal-fragment (ATF) of human uPA was provided by Drs. A. Mazar and J. Henkin (Abbott Laboratories, IL). This product can be prepared as described by Mazar et al. *Fibrinolysis*, 6, Suppl. 1, 49-55, 1992.

## **Binding Studies of Peptides using Biacore Technology**

25

### Real-time biomolecular interaction analysis (BIA)

Association rate constants ( $k_{\text{ass}}$ ), dissociation rate constants ( $k_{\text{diss}}$ ) and equilibrium binding constants ( $k_{\text{d}}$ ) for various synthetic peptides and receptor binding derivatives of human uPA were determined by surface plasmon resonance (R. Karlsson et al. (1991) *J. Immunol. Meths.* 145:229-240) using a BIAcore2000<sup>TM</sup> instrument (Pharmacia Biosensor, Uppsala, Sweden). Due to the low isoelectric point of uPAR ( $\text{pI} \approx 4.5$ ) terminal sialic acid residues were removed from its N-linked carbohydrates to facilitate chemical immobilisation on the sensor chip. This was accomplished by incubating purified suPAR (1

35 mg/ml in 50 mM phosphate, 150 mM NaCl, pH 7.4) with 5  $\mu\text{g/ml}$  neuraminidase for 4 hours

- at 37°C. Such desialylation did not alter the uPA binding kinetics of suPAR (M. Ploug et al. *J. Biol. Chem.*, Vol. 273, 22, 13933-13943, 1998). Coupling of the neuraminidase-treated suPAR to a BIAcore sensor chip was achieved by injection of 20 µg/ml suPAR in 10 mM sodium acetate pH 5.0 for 6 minutes at a flow rate of 5 µl per minute using a
- 5 carboxymethylated dextran matrix (CM5 sensor chip) preactivated with N-hydroxysuccinimide/N-ethyl-N'-[3-(diethyl-amino)propyl]carbodiimide according to the manufacturer's recommendations. Approximately 1,500-2,000 resonance units (RU) were immobilised by this procedure (corresponding to 1-2 ng suPAR/mm<sup>2</sup>).
- 10 Sensorgrams (RU versus time) were recorded (Figure 2) at a flow rate of 10 µl per min at 5°C using several different concentrations of the ligand (synthetic peptides, uPA, the amino-terminal fragment (ATF) of uPA, or the growth factor domain (GFD) of uPA) in the range of 10 to 1000 nM in running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA (pH 7.4) including 0.005% surfactant P-20). Stock solutions of peptides from combinatorial
- 15 chemistry were made in DMSO (10 mg peptide/ml DMSO). The sensor chip was regenerated at the end of each run by injection of 0.1 M acetic acid, 0.5 M NaCl. Data obtained from parallel mock coupled flow cells (subjected to the coupling procedure in the presence of buffer only) served as blank sensorgrams for subtraction of changes in bulk refractive index. The sensorgrams obtained were analysed by non-linear least squares
- 20 fitting using BIAevaluation 2.0 software (Pharmacia Biosensor, Uppsala, Sweden) assuming single-site association and dissociation models.

Biacore affinity measurements were made for the peptides described herein as well as for the reference peptides. The results are summarised in Table 2, and typical sensorgrams

25 are shown in Fig 2.

### Example 1

#### Identification of Critical Residues for Activity

- 30 The starting peptide sequence used was the peptide disclosed earlier by Chiron as Clone 20 (R.J. Goodson et al. (1994) *Proc. Nat. Acad. Sci. U.S.A.* 91:7129-7133). The peptide consists entirely of naturally occurring amino acids:

A E P M P H S L N F S Q Y L W Y T (AE78)

a) The effect of truncation of this peptide was first studied. Truncation was performed by (step-wise) elimination of sets of two amino acids from the C-terminal end and from the N-terminal end. By this procedure a 10-mer peptide was identified as the minimum sequence retaining good activity:

5

S L N F S Q Y L W S (AE68)

b) An alanine scan of this 10-mer was then performed in order to identify the functionally most important residues. The result obtained was:

10

S L N F S Q Y L W S

where the underlined residues are considered critical for the activity of the peptide. This finding furthermore suggested that 2 distinct sites exist in the preferred antagonist, which  
15 bind to 2 distinct sites in human uPAR.

With respect to the alanine scan performed, the exact experimental conditions are given in M. Ploug et al. (1998) *Biochemistry*, 37:3612-3622.

## 20 **Example 2**

### **Photochemical Identification of Interaction Sites**

The photoaffinity labeling of human uPAR was carried out as described in Ploug et al. (1998) *Biochemistry* 37:3612-3622.

25

Photoaffinity labeling confirmed that a two-site interaction occurs with the human uPAR molecule, and specifically that:

a) F interacts primarily with Arg53, and to a less extent with Leu66 loop 3 of uPAR  
30 Domain I.

b) W interacts with His251 in loop 3 of uPAR Domain III.

Thus, two more or less independently acting binding sites were identified, situated on separate structural domains. This composite binding site is thus possibly assembled through interdomain interactions.

### 5 Example 3

#### Higher Affinity Peptides from Combinatorial Chemistry

In the combinatorial chemistry studies, the following residues (underlined) were fixed as shown:

10



and a sub-library was synthesised which incorporated unnatural amino acids in the remaining positions. The unnatural amino acids were selected from: 18 D-amino acids: D-Ala, D-Ser, D-Thr, D-Tyr, D-Asp, D-Glu, D-Lys, D-His, D-Arg, D-Asn, D-Gln, D-Pro, D-Leu, D-Val, D-Ile, D-Met, D-Phe, D-Trp, O-benzyl-L-tyrosine, O-benzyl-L-hydroxyproline, N<sup>1</sup>-benzyl-L-histidine,  $\beta$ -2-naphthyl-L-alanine (abbreviated <sup>2</sup>nA),  $\beta$ -cyclohexyl-L-alanine (abbreviated Cha), D-phenylglycine as well as glycine.

20 The combinatorial peptide libraries were generated as described in e.g. S.E. Cwirla et al. (1997) *Science* 276:1696-1699. Thus, 1 gram of TentaGel S amine (Rapp Polymere) resin was washed thoroughly with dimethylformamide (DMF) and 5% diisopropylamine (DIEA) and finally with DMF. Standard Fmoc solid phase peptide synthesis was employed in the generation of the peptidylresin YLWS-Resin using 4 eq of Fmoc-amino acid, DIC and  
25 HOBt, respectively, during all coupling steps. The resin was then distributed equally into 25 reaction vessels and coupled with the above-mentioned unnatural amino acids.

After coupling overnight the 25 portions were combined and mixed thoroughly and subsequently the Fmoc group was removed and the resin was again distributed into 25  
30 portion and coupled with the above-mentioned unnatural amino acids to obtain the X-X-Y-L-W-S-resin. The peptidylresin X-X-Y-L-W-S-Resin, where X represents any of the 25 amino acids mentioned above, was coupled with Fmoc-Phe-OH using DIC and HOBt giving the peptidylresin F-X-X-Y-L-W-S-resin. This resin was again used in the divide, mix and recombine procedure, with the exception that small amount of resin was removed  
35 before each distribution step as shown in Table 1.

TABLE 1

Removal of resin during the (X)<sub>0-3</sub>-F-X-X-YLWS-resin synthesis

	X = 0	about 10,000 beads removed	(FXXYLWS-resin)
5	X = 1	about 100,000 beads removed	(FXXXYLWS-resin)
	X = 2	about 500,000 beads removed	(XXFXXYLWS-resin)
	X = 3	the remaining ~2.3 mill beads	(XXXFXXYLWS-resin)

After the final coupling all resin fractions were washed with DMF, deprotected with 20%  
 10 piperidine (PIP) in DMF and finally washed thoroughly with tetrahydrofuran (THF). A  
 solution of 85% trifluoroacetic acid (TFA), 5% thioanisole, 5% mercaptoethanol and 5%  
 phenol was added to the peptidylresin, (X)<sub>0-3</sub>-F-X-X-Y-L-W-S-resin, for 1.5 hours and  
 subsequently washed with THF and gradually with 10% AcOH in H<sub>2</sub>O. Finally the  
 peptidylresin was lyophilised.

15

The screening for activity was performed in the following way:

The library was incubated with streptavidin alkaline phosphatase diluted 1:10,000 (Sigma  
 (1mg/ml)) for 45 min in 0.05 M tris-HCl buffer pH 7.4, 0.25 M NaCl, 0.1% gelatine, 0.05%  
 20 Tween 20. After washing with tris washing buffer (0.05 M tris-HCl, pH 7.4, 0.05% Tween  
 20, 0.25 M NaCl) staining was accomplished with 5-bromo-4-chloro-3-indolylphosphate in  
 tris-HCl 0.05 M, pH 9.2, 0.1 M NaCl, 0.05 M MgCl<sub>2</sub> for 60 min. Active blue beads  
 interacting with streptavidin were removed from the library which was subsequently  
 washed with 8 M guanidiniumchloride and recycled for a second screening with murine  
 25 anti-uPAR monoclonal antibody R2 (Rønne et al, 1991, FEBS Lett. 288, pp 233ff) (mAb  
 (R2)) (biotinylated) diluted 1:200. As above, the active blue beads were removed and the  
 library recycled for a third screening by incubation with the target receptor, human  
 urokinase plasminogen receptor (uPAR) diluted 1:200 and adding mAb (R2) diluted 1:500  
 and streptavidin alkaline phosphatase diluted 1:20,000 (Sigma 1 mg/ml). Active blue beads  
 30 were isolated and analysed using a protein sequencer.

Based on the above screening six peptides interacting with uPAR were identified and  
 surprisingly they all contained a  $\beta$ -cyclohexyl-L-alanine in the position neighbouring the L-  
 phenylalanine.

35



Three of these peptides (AE100, dCha and AE 108) were synthesised as described in the section "Peptide synthesis", and the binding data for these peptides are shown in Table 2.

Thus, one of the high-affinity peptides found consisted of a 9-mer with the following  
5 sequence:

d Cha F s r Y L W S (dCha)

Subsequently it was found that changing the D-Asp to L-Asp i.e.

10

D Cha F s r Y L W S (AE105)

further improved binding. Clearly the peptide AE105 is a more interesting peptide than AE78, as it has a 48 fold slower off-rate.

15

The distance allowable between F and YLWS was studied by introduction of glycine (AE116) and  $\beta$ -alanine (AE 117) residues between D-arginine and L-tyrosine. It was found that the distance between F and YLWF in the discovered 9-mer was in fact critical for maintenance of high affinity human uPAR binding, since introduction of spacing residues  
20 abolished the binding (see Table 2).

#### The "Biacore Binding Assay"

Purified human suPAR (1 mg/ml in 50 mM phosphate, 150 mM NaCl, pH 7.4) was  
25 incubated with 5  $\mu$ g/ml neuraminidase for 4 hours at 37°C.

In order to couple the neuraminidase-treated suPAR to a BIAcore sensor chip, 20  $\mu$ g/ml suPAR in 10 mM sodium acetate (pH 5.0) was injected for 6 minutes at a flow rate of 5  $\mu$ l per minute using a carboxymethylated dextran matrix (CM5 sensor chip), thereby  
30 immobilising approximately 1,500-2,000 resonance units (RU), which correspond to 1-2 ng suPAR/mm<sup>2</sup>. The CM5 sensor chip was preactivated with N-hydroxysuccinimide/N-ethyl-N'-[3-(diethyl-amino)propyl]carbodiimide in accordance with the manufacturer's recommendations.

Surface plasmon resonance sensorgrams (RU versus time) were recorded at a flow rate of 10 µl per min at 5°C using several different concentrations of the ligand (i.e. the relevant peptide or GFD) in the range from 10 to 1000 nM in running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA (pH 7.4) including 0.005% surfactant P-20).

5

The sensor chip was regenerated at the end of each run by injection of 0.1 M acetic acid, 0.5 M NaCl.

10 Data from parallel mock coupled flow cells (subjected to the coupling procedure in the presence of buffer only) served as blank sensorgrams for subtraction of changes in bulk refractive index.

The obtained sensorgrams were analysed by non-linear least squares fitting using BIAevaluation 2.0 software (Pharmacia Biosensor, Uppsala, Sweden) assuming single-site  
15 association and dissociation models.

#### **Example 4**

##### **Modification of the Identified $\beta$ -Cyclohexyl-L-alanine-containing Sequence**

20 Based on the above-mentioned sequences, a number of analogues were synthesised and tested (AE106, AE107, LCha, AE109, AE110, AE114, AE115, AE130, AE131, and AE132 - see Table 2). These peptides had either activity of the same order of magnitude as AE105 or less than AE105.

#### **25 Example 5**

##### **Construction of Peptomer Libraries**

Synthesis of a peptidomimetic bead library comprising of a mixture of peptide-peptoid hybrids was accomplished by conventional peptoid chemistry using primary amines and  
30 bromoacetic acid (Zuckerman et al 1992, Østergaard and Holm 1997). At variable positions in the constrained motif of the library, diversity was created by iterative divide, couple, and pool approach of the solid-phase bead support. The peptidomimetic library was constrained according to the following motif: D[L-Cha]FsrXXXX, where the lower case letters are D-amino acids, capital letters are L-amino acids, and X is selected randomly  
35 among the following 12 amino acids and 13 amines: L-Tyr, L-Trp, L-Leu, L-Cha, D-Try, D-

- Leu, D-Cha, D-Phe, D-His,  $\beta$ -naphthyl-L-alanine, N<sub>im</sub>-benzyl-L-histidine, 1-aminoindane, tryptamine, diphenylethylamine, 1-aminonaphthalene, benzylamine, 2,3-dimethoxybenzylamine, 2-aminoethyl-2-pyridine, 2-(4-methoxy)-phenylethylamine, aminomethylcyclohexane, isobutylamine, 3,3-dimethylbutylamine, 1-butylamine and 2-methoxyethylamine. To reveal the chirality of a certain amino acid in a selected peptidomimetic during microsequencing, where both diastomers of that particular amino acid originally were present in the library, the L-amino acids were encoded with nor-leucine, whereas the corresponding D-amino acids were encoded with nor-valine.
- 10 The binding affinity of a number of analogues (AE124, AE125, AE126, AE128, AE129 - Table 2) was tested and AE124, AE125, AE126 and AE128 showed dissociation constants in the same order of magnitude as the best of the peptides. Analogue AE126 showed a relative dissociation constant approximating those of AE105 and AE110.

#### 15 **Example 6**

##### **Dimeric Peptide with Higher Affinity**

- Having identified an optimised 9-mer peptide for human uPAR binding, it was considered relevant to investigate whether an even further increase in effectiveness could be achieved by making multimeric constructs which could bind to more than one uPAR molecule at the same time.

To test this hypothesis, two dimers were synthesised as described in the section entitled "Peptide Synthesis". In the first dimer constructed (AE118) one glycine residue was inserted at the C-terminus of AE105 and a single lysine residue was employed as the scaffold. The second dimer (AE120) differed from the AE118 dimer in that a beta-alanine was attached to the C-terminus glycine in the peptide sequence being linked to the  $\alpha$ -amino group of the lysine scaffold. Thus, the structure of the two dimers were:

30                                    [DChaFsrYLWSG]<sub>2</sub>-K                                    (AE118)

$\alpha$ -[DChaFsrYLWSG $\beta$ A]- $\epsilon$ -[DChaFsrYLWSG]-K                                    (AE120)

- The dimers AE118 and AE 120 both had a considerably slower off-rate (about 5-7 times slower) than the monomer (AE105) (see Table 2).

**Example 7****Inhibition of uPA Binding to uPAR on the Surface of Human Carcinoma Cells**

5

The peptides were tested for their ability to inhibit binding of radio-labelled amino terminal fragment (ATF) of human uPA to monolayer cultures of human MDA-MB-231 breast cancer cells.

10 Cell binding experiments with  $^{125}$ I-labelled human ATF

MDA-MB-231 BAG cells were harvested by trypsin/EDTA treatment and added to 5% FCS supplemented medium in 24 well cluster plates at a cell density of 100,000 cells per well or as indicated otherwise. For background estimation, wells without cells were included in order to measure binding of  $^{125}$ I-labelled ligand to the plastic. Next day the adherent monolayers were washed twice in binding-buffer (0.1% bovine serum albumin (BSA; Fraction V, Sigma) and 5 mM Hepes in Eagles MEM supplemented with Glutamax-1, pH 7.4). Before binding experiments, the cells were subjected to acid treatment to liberate receptor-bound endogenous uPA. Each culture well was incubated for 3 min at 23°C with 200  $\mu$ L of 0.05 M glycine-HCl, 0.1 M NaCl, pH 3.0, after which 200  $\mu$ L of 0.5 M Hepes, 0.1 M NaCl pH 7.5 was added to neutralize the acid buffer. The wells were then emptied and washed once with binding buffer. Various competitors diluted in binding buffer were then added to the wells, followed by a pre-incubation at 37°C for 15 min. Finally,  $^{125}$ I-labelled ligand was added to each well at a final concentration of 1 nM. Cells were incubated at 25 °C for 60 min, and then washed three times in binding-buffer. Subsequently, the cells were lysed by addition of 1 N NaOH to the wells. Aliquots from the wells were finally measured for radioactivity on a  $\gamma$ -counter. The results are shown in Figure 3.

Results

30

The results of cell-binding experiments are shown in Figures 3 & 4 and the numerical values are compiled in Table 3. The monomers AE105 and AE 120 were more effective than the reference peptide AE78 in inhibiting radio-labelled human ATF binding to human carcinoma cell-surface uPAR, and the dimer AE118 was considerably more effective than

both, coming close to the potency of diisopropylfluorophosphate-inactivated human uPA (Fig. 3).

These cell binding experiments were done in the presence of 0.1% bovine serum albumin, but the effect of AE118 was also observed in the presence of 0.1% and even 10% fetal calf serum (Fig. 4). Thus the effect of AE118 on ligand binding to cell-surface uPAR can be maintained in the presence of a high background concentration of serum proteins, indicating favourable specificity for use in vivo (Fig. 4).

## 10 Example 8

### Biological Stability of the Dimeric Peptides AE118 and AE120

The inclusion of D-amino acids (instead of the natural L-amino acids) results in a peptide product which has non-natural residues involved in peptide bonds, and these bonds are not likely to be hydrolysed by endopeptidases. Thus, dimeric peptides containing D-amino acids and a blocked C-terminus, like AE118 and AE120, are expected to have greater biological stability, due to resistance to enzymatic hydrolysis.

#### Peptide stability in mouse serum ex vivo

20

Mouse blood was collected from anaesthetised mice (femal *nu/nu*-META/Bom (Bomholtgaard, Ry, Denmark)) by cardiac puncture. The blood samples were allowed to clot for 30 min at room temperature, followed by centrifugation at 1,000 x *g* for 10 min at 20°C, and the sera were sterile filtered using 0.8 µm/0.2 µm filters (Acrodisc PF, Gelman Science, MI) and then stored at 4°C until use. The peptide antagonists were added to fresh pooled mouse serum at the indicated concentrations (Figure 5) and incubated at 37°C for different periods, between 0 hours and 24 hours. Serum incubated samples were diluted 1:10 in binding buffer (0.1% bovine serum albumin and 5 mM Hepes in Eagles MEM supplemented with Glutamax-1, pH 7.4). Subsequently, cell binding experiments in the presence of <sup>125</sup>I-labelled human ATF were performed in duplicates as above, by pre-incubating MDA-MB-231 BAG cells at 37°C 15 min with the 1:10 diluted serum incubated samples ± peptides. In parallel, peptides diluted in binding buffer alone were included as controls.

AE118 and AE78 (for comparison) were incubated at 37°C in the presence of 10% mouse serum for different time intervals, up to 24 hours. The incubation mixtures were tested for their ability to inhibit binding of radio-labelled human uPA amino terminal fragment (ATF) to monolayer cultures of human MDA-MB-231 breast cancer cells, as described in Example

5 7.

Incubation of AE118 with mouse serum for 24 h produced only a slight reduction in its ability to inhibit ATF binding to cells. On the contrary, AE78 lost the majority of its inhibitory activity after incubation for 24 h with mouse serum (Fig. 6).

10

Thus AE118 is clearly more resistant than AE78 to proteolytic degradation in a complex biological fluid.

### Example 9

#### 15 **Pharmacokinetic experiments - Presence of peptides in mouse serum for a prolonged period**

Female *nu/nu*-META/Bom mice were injected intraperitoneally (i.p) with a solution of the peptide AE 120 (6.25 mg/ml in 0.5% (w/v) poloxamer 237 in PBS, pH 7.3) at a dose of 62.5  
20 mg/kg mouse weight. At different time points between 1 hour and 24 hours, blood was collected from anaesthetised mice by cardiac puncture. The blood samples were allowed to clot for 30 min at room temperature, followed by centrifugation at 1,000 x g for 10 min at 20 °C, and the sera were stored frozen at -80 °C prior to assay. The presence of functional peptide antagonists in serum was determined by diluting the serum samples 1:10 with  
25 binding buffer (0.1% bovine serum albumin and 5 mM Hepes in Eagles MEM supplemented with Glutamax-1, pH 7.4) and analysing the samples in cell binding experiments with MDA-MB-231 BAG cells and <sup>125</sup>I-labelled human ATF as described in Example 7. As an internal standard, AE120 was added exogenously to pooled mouse sera at final concentrations of 12.5 nM, 25 nM, 125 nM and 250 nM.

30

The results (see Fig. 7) show that the peptide antagonist retained its activity in mouse serum for several hours. This result suggests that the peptide antagonists according to the present invention may be potential drugs in their own right, especially because of their low molecular weight and favourable solubility in aqueous media.

35

**Example 10****Mouse Tolerance of Peptide Dosing**

Mice were given a series of 4 intraperitoneal injections, spaced 8 hours apart, of peptide  
5 AE120 dispersed in a poloxamer formulation containing 2.9% DMSO. Each injection  
delivered 1.38 mg of peptide in 200  $\mu$ L of formula, so that each mouse received a total of  
5.52 mg over the 24-hour study period. Throughout the time of treatment there was no  
visible sign of any effect on the behaviour or well-being of the mice, so that the peptide at  
this dose was considered to be free of any significant toxicity.

10

**Example 11****Species specificity of the uPA interaction with uPAR.**

The species specificity of the interaction between the peptide antagonist AE120 and  
15 recombinant human versus mouse suPAR was measured by surface plasmon resonance  
in which the branched peptide antagonist AE120 was immobilised on the sensor chip via  
one of its two amino groups using traditional amine coupling chemistry. The binding of 200  
nM purified human suPAR and mouse suPAR was measured in real times by recording the  
changes in the surface plasmon resonance. Human suPAR was found to bind with very  
20 high affinity, whereas purified mouse suPAR did not interact to any significant degree  
(Figure 8).

**Example 12**

**Sequence comparison of the relevant amino acids subjected to alanine mutagenesis**  
25 **in human uPAR to the corresponding residues of hamster, mouse, rat and bovine**  
**uPAR**

As shown in Figure 8 we have demonstrated that the present peptide antagonists of the  
uPA-uPAR exhibit a very stringent species specificity, which then imposes limitations on  
30 which experimental animal model can be used to demonstrate the anti-metastatic and/or  
anti-invasive properties of the peptide antagonist. Any involvement of mouse stromal  
uPAR in human xenograft tumour invasion and metastasis performed in nude mice would  
therefore remain uninhibited during the treatment with AE120.

The functional epitope for antagonist binding was investigated by single site alanine scanning mutagenesis and revealed that His<sup>249</sup>, Asp<sup>254</sup> and Phe<sup>256</sup> contribute by far the most to the free energy of binding. Among these, it was revealed that His<sup>249</sup> in human uPAR contributes most significantly to the free energy of antagonist binding, but not to the binding of the natural ligand (human uPA) and that only His<sup>249</sup> is changed when comparing the human sequence with the corresponding mouse and hamster sequence. Residues in human uPAR important for the free energy of human uPA binding are high-lighted in Figure 9 by asterisks, whereas those important for binding of the peptide antagonist AE105 are boxed with a black frame. The residue in human uPAR, which is critical for antagonist binding and has been non-conservatively substituted in all other species (His<sup>249</sup>), is marked with an arrow. Also shown is the ability of uPAR from different species to bind to human uPA and whether this binding can be inhibited by the peptide antagonist.

Based on these findings, we propose a method to circumvent the species-barrier by genetically engineering a mouse or hamster uPAR in such a way that the binding to the natural ligand uPA is unchanged but this binding is now rendered susceptible to inhibition by the peptide antagonists. One option to circumvent this species barrier would be to replace the mouse gene for uPAR by a mutated gene by gene targeting (Hanks et al 1995, Science 269, 679-682) in which Gly<sup>272</sup> has been changed to a His by the established "gene knock-out/ gene knock-in" technology. In such a system we will possibly be able to study the inhibitory effect of the "species specific" peptide antagonists on mouse tumours.

It would therefore be possible to use a gene-targeted mouse as a model system for the intervention of cancer invasion and/or metastasis using the described "species specific" antagonists. Such a gene-targeted mouse would be unlikely to have significantly altered endogenous function of uPAR; i.e. cells would probably bind mouse uPA, but it is possible that this binding would then be rendered susceptible to inhibition by the peptide antagonist. This would greatly help to demonstrate the efficacy of the antagonists in an experimental human tumour in mice.

30

### Example 13

**Dissociation rate constants for the interaction between an immobilised peptide antagonist and various suPAR variants carrying single-site mutations.**



A linear derivative of the peptide antagonist AE105 having a 5 amino acid residue extension at the NH<sub>2</sub>-terminus (KGSGGD-Cha-srYLWS) was immobilised via its NH<sub>2</sub> terminal lysine residue to the sensor chip by amine coupling chemistry. Binding of various purified single-site suPAR mutants was measured in real-time using surface plasmon resonance for 8 different concentrations in the range 200 nM to 2 nM. The mean of the dissociation rate constants along with its standard deviation are shown in Figure 10 for each individual suPAR mutant.

TABLE 2

Summary of off-rates determined by Biacore technology for various peptides selected by combinatorial chemistry

Code	Sequence	$k_{\text{diss}}$ ( $\text{sec}^{-1}$ )	Relative $k_{\text{diss}}^{1)}$
AE68 <sup>2)</sup>	SLNFSQYLWS	$12.9 \times 10^{-3}$	92.1
dCha	d- <u>Cha</u> -F-s-r-Y-L-W-S	$0.68 \times 10^{-3}$	4.9
AE100	e- <u>Cha</u> -F-s-y-Y-L-W-S	$0.56 \times 10^{-3}$	4.0
AE108	t- <u>Cha</u> -F-s-r-Y-L-W-S	$0.48 \times 10^{-3}$	3.4
AE105	D- <u>cha</u> -F-s-r-Y-L-W-S	$0.21 \times 10^{-3}$	1.5
AE116	D- <u>cha</u> -F-s-r-G-Y-L-W-S	no binding	>>100
AE117	D- <u>cha</u> -F-s-r- $\beta$ A-Y-L-W-S	no binding	>>100
AE106	D- <u>Cha</u> -F-S-r-Y-L-W-S	$2.63 \times 10^{-3}$	18.7
AE107	d- <u>Cha</u> -F-S-r-Y-L-W-S	$8.84 \times 10^{-3}$	63.1
Lcha	S-L- <u>Cha</u> -F-s-Q-Y-L-W-S	$3.49 \times 10^{-3}$	24.9
AE109	d- <u>Cha</u> -F-s-r-Y-L <sup>2</sup> -nA-S	$2.05 \times 10^{-3}$	14.6
AE110	D- <u>Cha</u> -F-s-R-Y-L-W-S	$0.28 \times 10^{-3}$	2.0
AE114	D- <u>Cha</u> -F-s-r-Y-L <sup>1</sup> -nA-S	$0.59 \times 10^{-3}$	4.2
AE115	e- <u>Cha</u> -F-s-Y-Y-L-W-S	$1.67 \times 10^{-3}$	11.9
AE118	[D <u>Cha</u> FsrYLWSG] <sub>2</sub> -K	$0.04 \times 10^{-3}$	0.28
AE120	$\alpha$ -[D <u>Cha</u> FsrYLWSG $\beta$ A]- $\epsilon$ -[D <u>Cha</u> FsrYLWSG]-K	$0.03 \times 10^{-3}$	0.21
AE130	D- <u>Cha</u> -F-s-r-L-L-W-h	$0.51 \times 10^{-3}$	3.6
AE132	D- <u>Cha</u> -F-s-r-Cha-L-W-I	$0.53 \times 10^{-3}$	3.8
AE131	D- <u>Cha</u> -F-s-r-Y-L-Nal-h	$0.56 \times 10^{-3}$	4.0
AE78 <sup>3)</sup>	AEPMPHSLNFSQYLWYT	$10.1 \times 10^{-3}$	72.1
AE104	a-r-F-h-h-Y-L-W-S	no binding	>>100
AE111 <sup>4)</sup>	L-N-F-s-Q-Y-L-W-S	$1.05 \times 10^{-3}$	7.5
AE112	D-F-F-s-r-Y-L-W-S	$1.81 \times 10^{-3}$	12.9
AE113	D-N-F-s-r-Y-L-W-S	no binding	>>100
AE124	D- <u>Cha</u> -F-s-r-DMB-f-TRA-MEA <sup>5)</sup>	$5.61 \times 10^{-3}$	40.0
AE125	D- <u>Cha</u> -F-s-r-DMB-f-Bzl-MEA	$0.74 \times 10^{-3}$	5.3
AE126	D- <u>Cha</u> -F-s-r-DMB-f-AMN-MEA	$0.37 \times 10^{-3}$	2.6

Code	Sequence	$k_{\text{diss}}$ ( $\text{sec}^{-1}$ )	Relative $k_{\text{diss}}^1)$
AE128	D-Cha-F-s-r-MEA-DMB-f-AMN	no binding	>>100
AE129	D-Cha-F-s-r-DMB-f-DMB-I	$0.54 \times 10^{-3}$	3.9
pro-uPA		$0.10 \times 10^{-3}$	0.72
ATF		$0.12 \times 10^{-3}$	0.86
GFD		$0.14 \times 10^{-3}$	1.0

- 1) Relative  $k_{\text{diss}} = k_{\text{diss}}(\text{peptide})/k_{\text{diss}}(\text{GFD})$ .
- 2) Disclosed in Ploug et al. (1998) *Biochemistry* 37:3612-3622.
- 3) Disclosed in Godson et al. (1994) *Proc. Nat. Acad. Sci. U.S.A.* 91:7129-7133 as the  
5 so-called "Clone 20".
- 4) Equivalent to Chiron's "optimal" in WO 97/05257.
- 5) DMB N-(2,3-dimethoxybenzyl)glycine; TRA is N-(3-indolyethyl)glycine, made from  
Tryptamine; MEA is N-(2-methoxyethyl)glycine made from methoxyethylamine; Bzl  
is N-benzylglycine made from benzylamine; AMN is N-(methylnaphthalyl)glycine  
10 made from aminomethylnaphthaline.

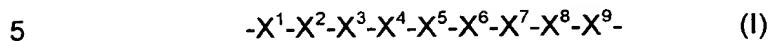
TABLE 3

Effect of various peptide antagonists in the inhibition of the binding of ATF to mono-layer cultures of human MDA-MB-231 breast cancer cells.

Code	Sequence	$\text{IC}_{50}$ (nM)
AE118	[D <u>Ch</u> aFsrYLWSG] <sub>2</sub> -K	2
AE78	AEPMPHSLNFSQYLWYT	200
AE105	D <u>ch</u> aFsrYLWS	10
DFP-uPA		1

## CLAIMS

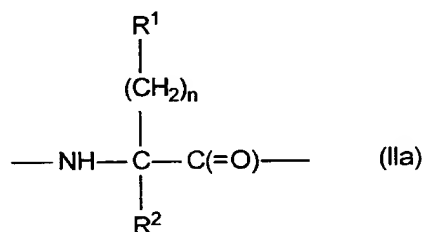
1. A peptide comprising at least one peptide fragment of the general formula I:



wherein

$X^1$ ,  $X^5$ ,  $X^6$ ,  $X^7$  and  $X^9$  are independently selected from amino acids and  $X^1$  is the N-terminal  
10 amino acid of the fragment and  $X^9$  is the C-terminal amino acid of the fragment;

$X^2$  is selected from the group consisting of amino acids of the general formula IIa

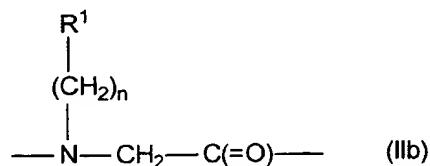


15 wherein n is an integer in the range from 0 to 3;  $R^1$  is selected from the group consisting of optionally substituted five-, six- and seven-membered non-aromatic rings;  $R^2$  is selected from the group consisting of hydrogen and  $C_{1-4}$ -alkyl; or,

$R^1$  and  $R^2$  together with the carbon atom to which they are bound form an optionally  
20 substituted cyclopentyl, cyclohexyl, cycloheptyl or decahydronaphthalenyl ring;

and

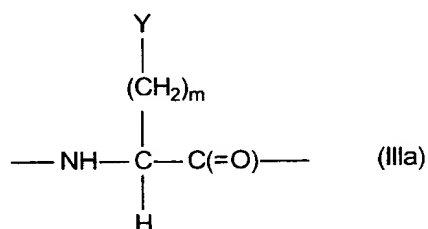
N-substituted amino acids of the general formula IIb



5 wherein n and R<sup>1</sup> are as defined above;

X<sup>3</sup> and X<sup>8</sup> are each independently selected from the group consisting of amino acids having hydrophobic side chains and amino acids having hydrophobic N-substituents;

10 X<sup>4</sup> is selected from the group consisting of amino acids of the general formula IIIa

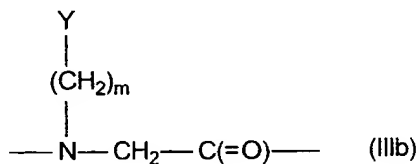


wherein m is an integer in the range from 1 to 3, and Y is selected from the group  
15 consisting of OH, SH, NH<sub>2</sub>, CONH<sub>2</sub>, COOH and OPO<sub>3</sub>H;

and

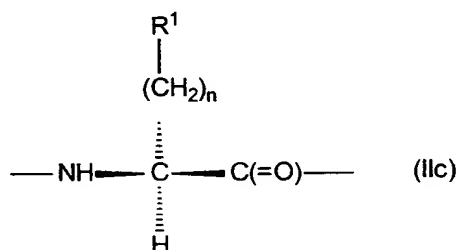
N-substituted amino acids of the general formula IIIb

20



wherein m and Y are as defined above.

2. A peptide according to claim 1, where the peptide, when tested in the Biacore Binding Assay described herein, has a dissociation rate constant ( $k_{\text{diss}}$ ) relative to GFD of at the most 5.0.
- 5 3. A peptide according to claim 1 or 2, wherein the peptide, when tested in the uPA/uPAR Binding Assay described herein, is able to inhibit the binding of radio-labelled ATF to cell-surface uPAR by 50% at a concentration of at the most 50 nM.
4. A peptide according to any of the preceding claims, wherein  $X^2$  is selected from amino  
10 acids of the general formula IIa, wherein n is 1 or 2 and  $R^2$  is H.
5. A peptide according to claim 4, wherein  $X^2$  is selected from L-amino acids of the general formula IIc



15

wherein n is 1 or 2 and  $R^1$  is selected from the group consisting of optionally substituted five-, six- and seven-membered non-aromatic rings.

20

6. A peptide according to claim 5, wherein n is 1.

7. A peptide according to claim 6 where  $R^1$  is selected from the group consisting of cyclopentyl, cyclohexyl and cycloheptyl.

25

8. A peptide according to claim 7, wherein  $R^1$  is cyclohexyl.

9. A peptide according to claim 1, wherein  $X^2$  is selected from N-substituted amino acids of the general formula IIb, wherein n is 1 or 2.

30

10. A peptide according to claim 9, wherein n is 1.

11. A peptide according to claim 10 wherein  $R^1$  is selected from the group consisting of cyclopentyl, cyclohexyl and cycloheptyl.
12. A peptide according to claim 11, wherein  $R^1$  is cyclohexyl.
- 5
13. A peptide according to any of the preceding claims, wherein the hydrophobicity constant ( $\pi$ ), as defined herein, of the side chain or the N-substituent of the amino acid in the  $X^3$  position is at least 0.5, preferably at least 1.0, such as at least 1.5, e.g. at least 2.0.
- 10 14. A peptide according to any of the preceding claims, wherein the hydrophobicity constant ( $\pi$ ), as defined herein, of the side chain or the N-substituent of the amino acid in the  $X^8$  position is at least 0.5, preferably at least 1.0, such as at least 1.5, e.g. at least 2.0.
- 15 15. A peptide according to claim 13 or 14, wherein  $X^3$  and  $X^8$  are each independently selected from amino acids with aromatic side chains or aromatic N-substituents.
16. A peptide according to claim 15, wherein  $X^3$  and  $X^8$  are each independently selected from amino acids with aromatic side chains.
- 20 17. A peptide according to claim 15, wherein  $X^3$  and  $X^8$  are each independently selected from the group consisting of D- and L-phenylalanine, D- and L-tryptophan, D- and L-tyrosine, D- and L-histidine,  $\beta$ -2-naphthyl-L-alanine,  $\beta$ -2-naphthyl-D-alanine,  $\beta$ -1-naphthyl-L-alanine,  $\beta$ -1-naphthyl-D-alanine, N-(2,3-dimethoxybenzyl)glycine, N-(3-indolyloethyl)glycine, N-benzylglycine, N-(methylnaphthalyl)glycine, N-(2,2-diphenylethyl)glycine, N-(indanyl)glycine, N-(2-ethyl-2-pyridinyl)glycine and N-(4-methoxyphenylethyl)glycine.
- 25
18. A peptide according to claim 17, wherein  $X^3$  and  $X^8$  are each independently selected from the group consisting of D- and L-phenylalanine, D- and L-tryptophan, D- and L-tyrosine, D- and L-histidine,  $\beta$ -2-naphthyl-L-alanine,  $\beta$ -2-naphthyl-D-alanine,  $\beta$ -1-naphthyl-L-alanine and  $\beta$ -1-naphthyl-D-alanine.
- 30
19. A peptide according to claim 18, wherein  $X^3$  is L-phenylalanine.

20. A peptide according to claim 17 where X<sup>8</sup> is selected from the group consisting of L-tryptophan, N-(2,3-dimethoxybenzyl)glycine, N-(3-indolylethyl)glycine, N-benzylglycine, N-(methylnaphthalyl)glycine, N-(2,2-diphenylethyl)glycine, N-(indanyl)glycine, N-(2-ethyl-2-pyridinyl)glycine and N-(4-methoxyphenylethyl)glycine.

5

21. A peptide according to claim 20, wherein X<sup>8</sup> is selected from the group consisting of L-tryptophan, N-benzylglycine, N-(methylnaphthalyl)glycine and N-(2,3-dimethoxybenzyl)glycine.

10 22. A peptide according to any of the preceding claims, wherein X<sup>4</sup> is selected from the group consisting of L-serine, D-serine, L-cysteine, D-cysteine, N-(hydroxymethyl)glycine and N-(methylthiol)glycine.

23. A peptide according claim 22, wherein X<sup>4</sup> is selected from the group consisting of L-  
15 serine, D-serine, L-cysteine and D-cysteine.

24. A peptide according to claim 23, wherein X<sup>4</sup> is D-serine.

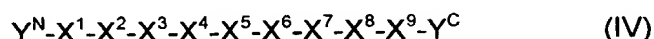
25. A peptide according to claim 1, wherein the peptide fragment is selected from the group  
20 consisting of dChaFsrYLWS, SLChaFsQYLWS, eChaFsyYLWS, DChaFsrYLWS, DChaFsRYLWS, dChaFsRYLWS, tChaFsrYLWS, dChaFsrYL<sup>2</sup>nAS, DChaFsRYLWS, DChaFsrYL<sup>1</sup>nAS, eChaFsYYLWS, D-Cha-F-s-r-L-L-W-h, D-Cha-F-s-r-Cha-L-W-I, D-Cha-F-s-r-Y-L-Nal-h, D-Cha-F-s-r-DMB-f-TRA-MEA, D-Cha-F-s-r-DMB-f-Bzl-MEA, D-Cha-F-s-r-DMB-f-AMN-MEA and D-Cha-F-s-r-DMB-f-DMB-I

25

wherein Cha designates β-cyclohexyl-L-alanine, <sup>1</sup>nA designates β-1-naphthyl-L-alanine, <sup>2</sup>nA designates β-2-naphthyl-L-alanine, capital letters designate L-amino acids, lower case letters designate D-amino acids, βA designates β-alanine, DMB designates N-(2,3-dimethoxybenzyl)glycine, TRA designates N-(3-indolylethyl)glycine, MEA designates N-(2-methoxyethyl)glycine, Bzl designates N-benzylglycine and AMN designates N-  
30 (methylnaphthalyl)glycine.



26. A peptide according to claim 1, having the general formula IV



5 wherein  $Y^N$  is an N-terminal group,  $Y^C$  is a C-terminal group, and  $X^1\text{--}\dots\text{--}X^9$  is the peptide fragment I as defined in claim 1.

27. A peptide according to claim 26, wherein the N-terminal group ( $Y^N$ ) is selected from the group consisting of a peptide radical having 1-7 amino acids, hydrogen,  $C_{1-4}$ -alkyl,  $C_{1-4}$ -alkylcarbonyl, optionally substituted arylcarbonyl and optionally substituted  
10 heteroarylcarbonyl.

28. A peptide according to claim 26 or 27, wherein the C-terminal group ( $Y^C$ ) is selected from the group consisting of a peptide radical having 1-7 amino acids, hydroxy,  $C_{1-4}$ -alkoxy, amino,  $C_{1-4}$ -alkylamino, optionally substituted arylamino and optionally substituted  
15 heteroarylamino, or  $Y^C$  designates a -linker-(peptide having 1-7 amino acids) moiety.

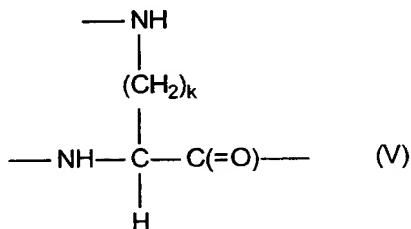
29. A peptide according to any of claims 26-28, wherein said peptide has a molecular weight of at the most 3,000.

20

30. A peptide according to claim 1, which comprises more than one peptide fragment of the general formula I.

31. A peptide according to claim 30, wherein each of the peptide fragments are attached to  
25 a common scaffold.

32. A peptide according to claim 31, wherein the scaffold is an L- or D-diamino acid of the general formula V



30

wherein k is an integer in the range from 1 to 6, preferably in the range from 2 to 5, such as 3 or 4.

33. A peptide according to claim 32, wherein the scaffold is selected from the group  
5 consisting of L-lysine, D-lysine, L-ornithine and D-ornithine.

34. A peptide according to claim 33, wherein the scaffold is L-lysine.

35. A peptide according to claim 32, wherein one or more of the peptide fragments are  
10 linked to the scaffold via one or more  $\omega$ -amino acid linkers of the general formula VI



wherein j is an integer in the range from 1 to 6, preferably in the range from 1 to 4, such as  
15 in the range from 1 to 3, e.g. 1 or 2.

36. A peptide according to claim 35, which is selected from the group consisting of  
 $\alpha$ -[DChaFsrYLWSG]- $\epsilon$ -[DChaFsrYLWSG]-L-lysine,  $\alpha$ -[DChaFsrYLWSG $\beta$ A]- $\epsilon$ -  
[DChaFsrYLWSG]-L-lysine, and  $\alpha$ -[DChaFsrYLWS $\beta$ A]- $\epsilon$ -[DChaFsrYLWS]-lysine.  
20

37. A peptide according to claim 30, wherein the scaffold has a dendritic structure carrying  
a plurality of amino and/or carboxylic acid groups.

38. A pharmaceutical composition comprising a peptide according to any of the claims 1-  
25 37.

39. A peptide according to any of the claims 1-37 for use as a medicament.

40. The use of a peptide according to any of the claims 1-37 for the manufacture of a  
30 medicament for the treatment of cancer.

41. A method for selecting a peptide antagonist which is suitable for preventing or counter-  
acting localized extracellular proteolytic activity of plasmin in a human by inhibiting the acti-

vation of plasminogen to plasmin by inhibiting the binding of a receptor-binding form of uPA to a uPAR in the human, the method comprising

providing a modified uPAR of a non-human mammalian species, said modified uPAR being  
5 modified in a manner which renders it capable of being antagonized by a peptide antagonist according to any of claims 1-37 while retaining its capability of binding to a receptor-binding form of uPA of said mammalian species substantially unchanged,

in a model system for assessing antagonism of uPA/uPAR binding and comprising said  
10 modified uPAR carried by cells of the non-human mammalian species as well as a receptor-binding form of uPA of the species, subjecting a panel of peptides to assessment in the model system and selecting, as peptide antagonists, such peptides among the panel of peptides which, in the model system, result in a degree of antagonism of the binding of the uPA to the modified uPAR which is equal to or not less than the degree of antagonism  
15 obtained by using an ATF-fragment of said uPA at a concentration of one-tenth of the concentration of the peptide antagonist.

42. A method according to claim 41, wherein said model system comprises one or more of the following test systems:

20

- 1) a screening assay in which the possible inhibition of uPA/uPAR interaction by the peptide antagonist is determined by adding the peptide antagonist to the system comprising the modified uPAR and solubilized uPA, uPA bound to uPAR being detected by being labelled or by means of a labelled anti-uPA antibody, or adding  
25 the substance to a system comprising immobilized uPA and solubilized uPAR, uPAR bound to uPA being detected by being labelled or by means of a labelled anti-uPAR antibody,
- 2) an assay in which the possible inhibition of uPA/uPAR interaction by the substance is determined by adding the substance to a system comprising uPAR and  
30 radiolabelled uPA or a derivative thereof, cross-linking any uPAR bound to uPA and detecting any cross-linked product by SDS PAGE and autoradiography,
- 3) an assay in which the possible inhibition of binding of uPA to uPAR on the surface  
35 of cultured cells is determined by adding the substance to a system comprising ra-

diolabelled uPA or a derivative thereof and cells carrying uPAR, and detecting any uPA or derivative binding to uPAR by gamma counting of the cells,

- 4) an assay in which the possible inhibition of cell surface plasminogen activation by receptor-bound exogenous pro-uPA is determined by adding the substance to cells carrying uPAR and subsequently adding pro-uPA, followed by measurement of plasmin generation on the cell surface,
  - 5) an assay in which the possible inhibition of cell surface plasminogen activation by receptor-bound endogenous pro-uPA is determined by incubating cells carrying uPAR and producing pro-uPA with the substance, followed by measurement of plasmin generation on the cell surface,
  - 6) an assay in which human or non-human tumour cells are inoculated into an immunocompromised mouse and recovered from the mouse after treatment with the peptide antagonist, followed by measurement of the reduction of bound uPA on the tumour cell surface using flow cytometric methods,
  - 7) administering a substance which has been established to inhibit uPA/uPAR interaction to a immunodeficient mouse which also lacks a functional gene for uPAR, said mouse being susceptible to invasion or metastasis by human or non-human tumour cells and said mouse being inoculated with human or non-human tumour cells which are known to invade and/or metastasize in the presence of uPA and uPAR and which are capable of invasion and/or metastasis in the mouse, and measuring the inhibition of the invasion and/or metastasis of the human or non-human tumour cells in the mouse.
43. A method according to claim 41 or claim 42, wherein the modified uPAR is a non-human uPAR modified by substituting an amino acid or amino acids thereof.
44. A method according to claim 43, wherein the amino acid or amino acids of the non-human uPAR substituted is/are selected from the group of amino acid residues corresponding to the amino acid residues His<sup>249</sup>, Ser<sup>254</sup> and Asn<sup>256</sup> of the human uPAR sequence.

45. A method according to claim 43, wherein the amino acid of the non-human uPAR substituted corresponds to the amino acid residue His<sup>249</sup> of the human uPAR sequence.
46. A method according to claim 45, wherein the amino acid of the non-human uPAR substituted corresponding to amino acid residue His<sup>249</sup> of the human uPAR sequence is substituted with the amino acid His.
47. A method according to any of claims 41-46, wherein the non-human uPAR is selected from the group consisting of murine uPAR, hamster uPAR, rat uPAR, dog uPAR, cat uPAR, bovine uPAR, rabbit uPAR and non-human primate uPAR.
48. A method according to claim 47, wherein the non-human uPAR is a murine uPAR in which Gly<sup>272</sup> of the murine uPAR has been substituted with His<sup>272</sup>.
49. A method according to any of claims 41-48, wherein the modified non-human uPAR has been generated by gene knock-out/ gene knock-in technology.
50. A method according to any of claims 41-49, wherein the model system comprises human tumour cells in an environment of non-human cells.
51. A method according to any of claims 41-49, wherein the model system comprises non-human tumour cells in an environment of non-human cells.
52. A method according to any of claims 41-49, wherein the model system comprises non-human primate tumour cells in an environment of non-human primate cells.
53. A method according to any of claims 49-52, wherein the modified uPAR is a uPAR the natural counterpart of which is associated with the particular tumour cells.
54. A method according to any of claims 49-52, wherein the modified uPAR is a uPAR the natural counterpart of which is associated with the environment of non-human cells.
55. A method according to any of claims 49-52, wherein the modified uPAR is a uPAR the natural counterpart of which is associated with both the particular tumour cells and the environment of the non-human cells.

56. A method according to any of claims 53-55, wherein the uPA is supplied by the particular tumour cells.

5 57. A method according to any of claims 53-55, wherein the uPA is supplied by the environment of non-human cells.

58. A method according to any of claims 41-57, wherein the model system is a transgenic mouse.

Figure 1

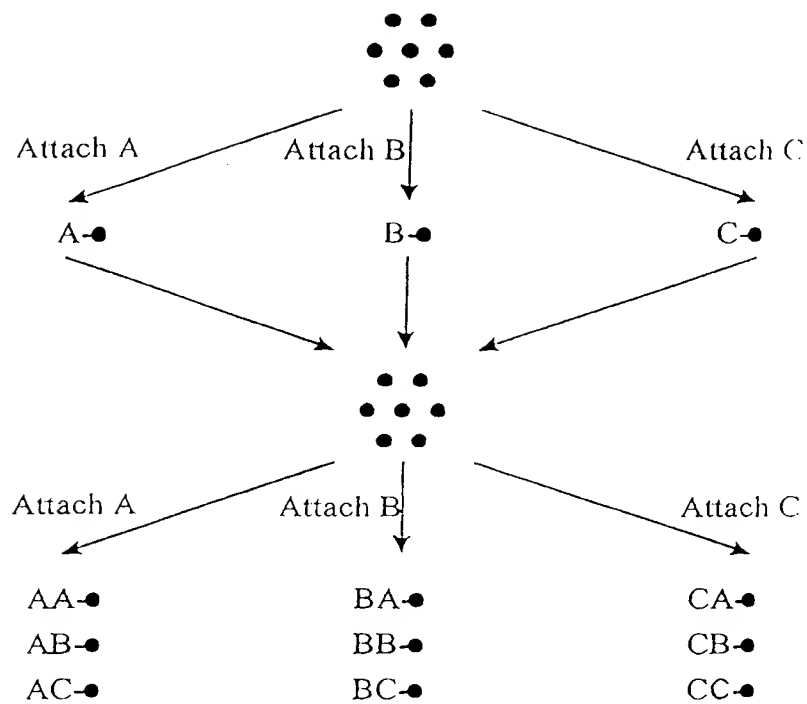


Figure 2

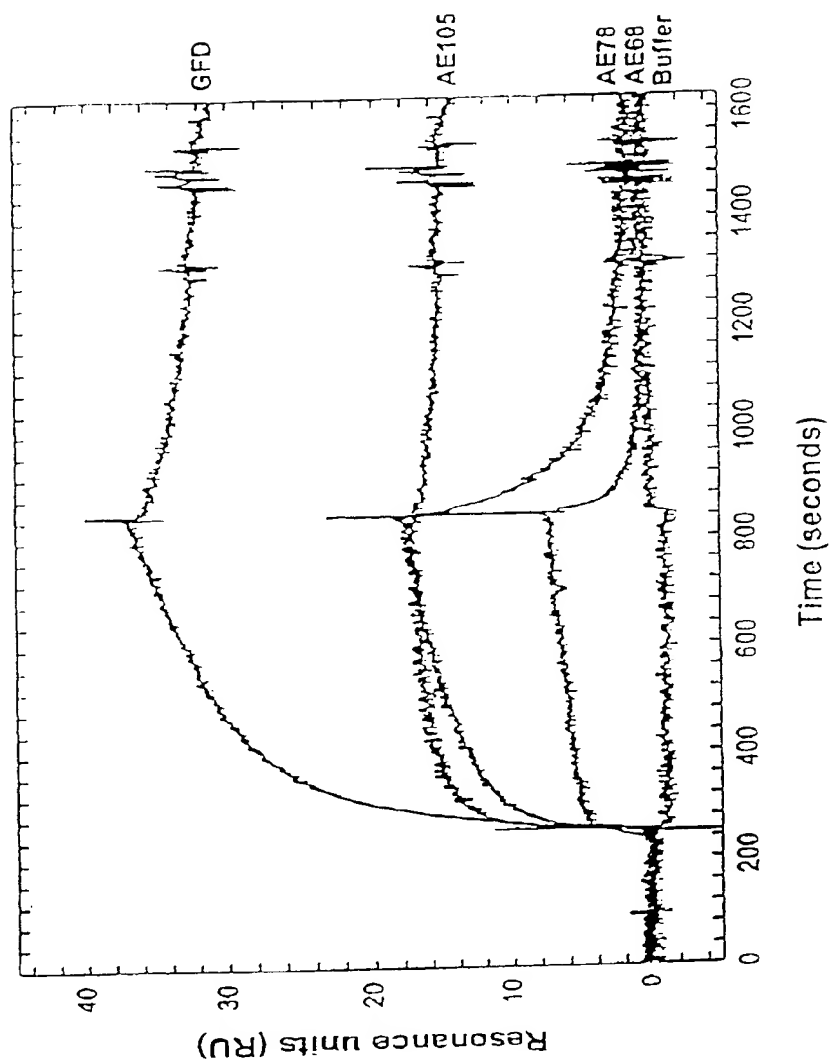




Figure 3

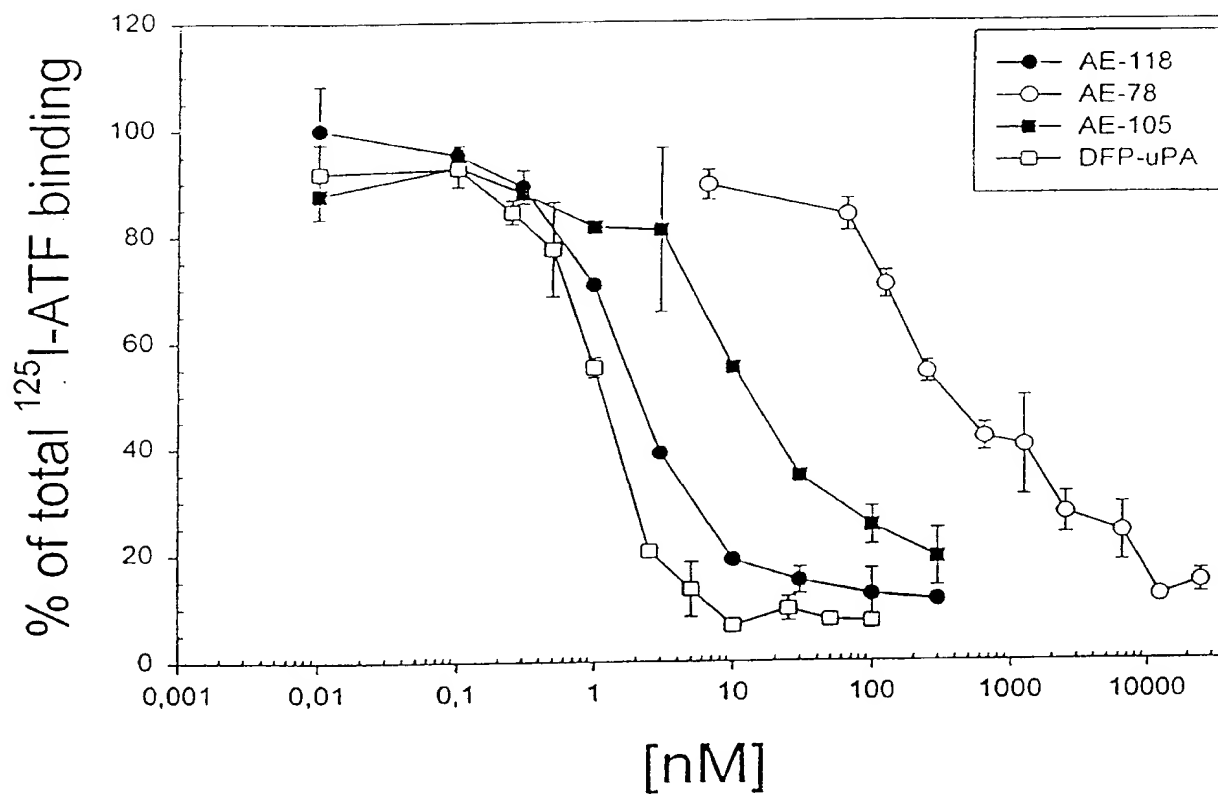


Figure 4

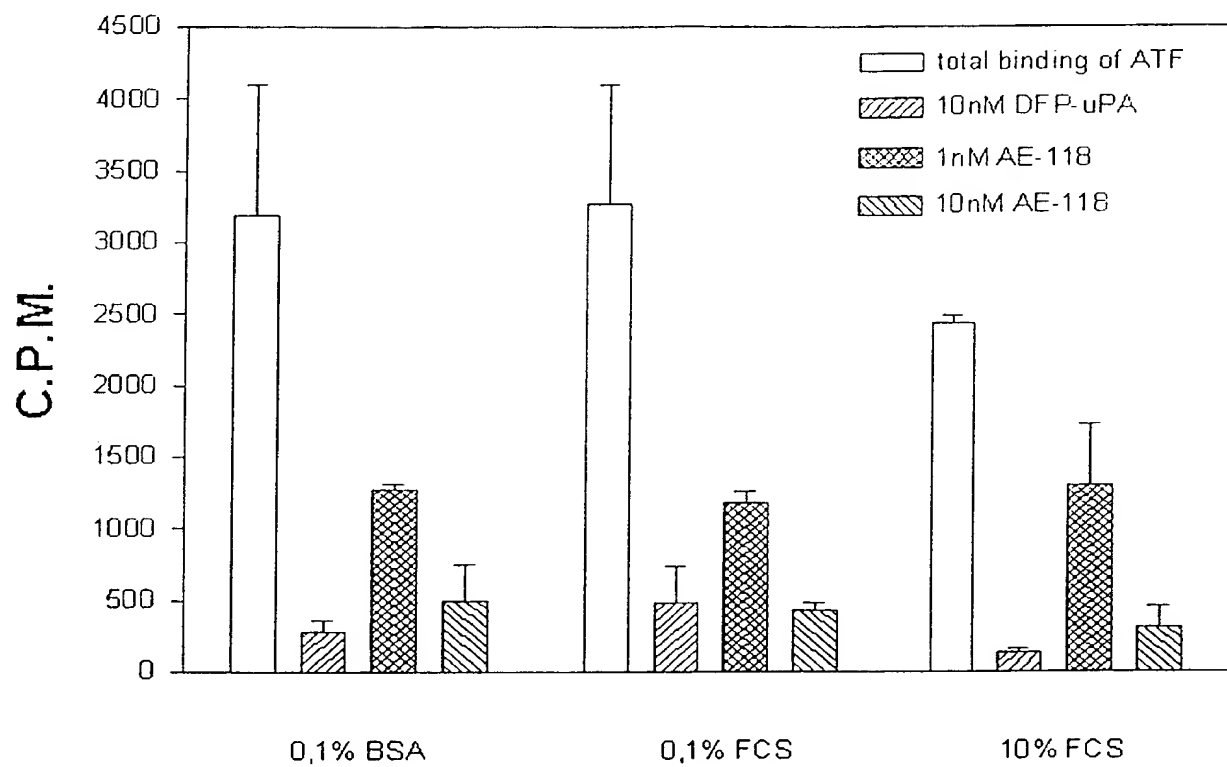


Figure 5

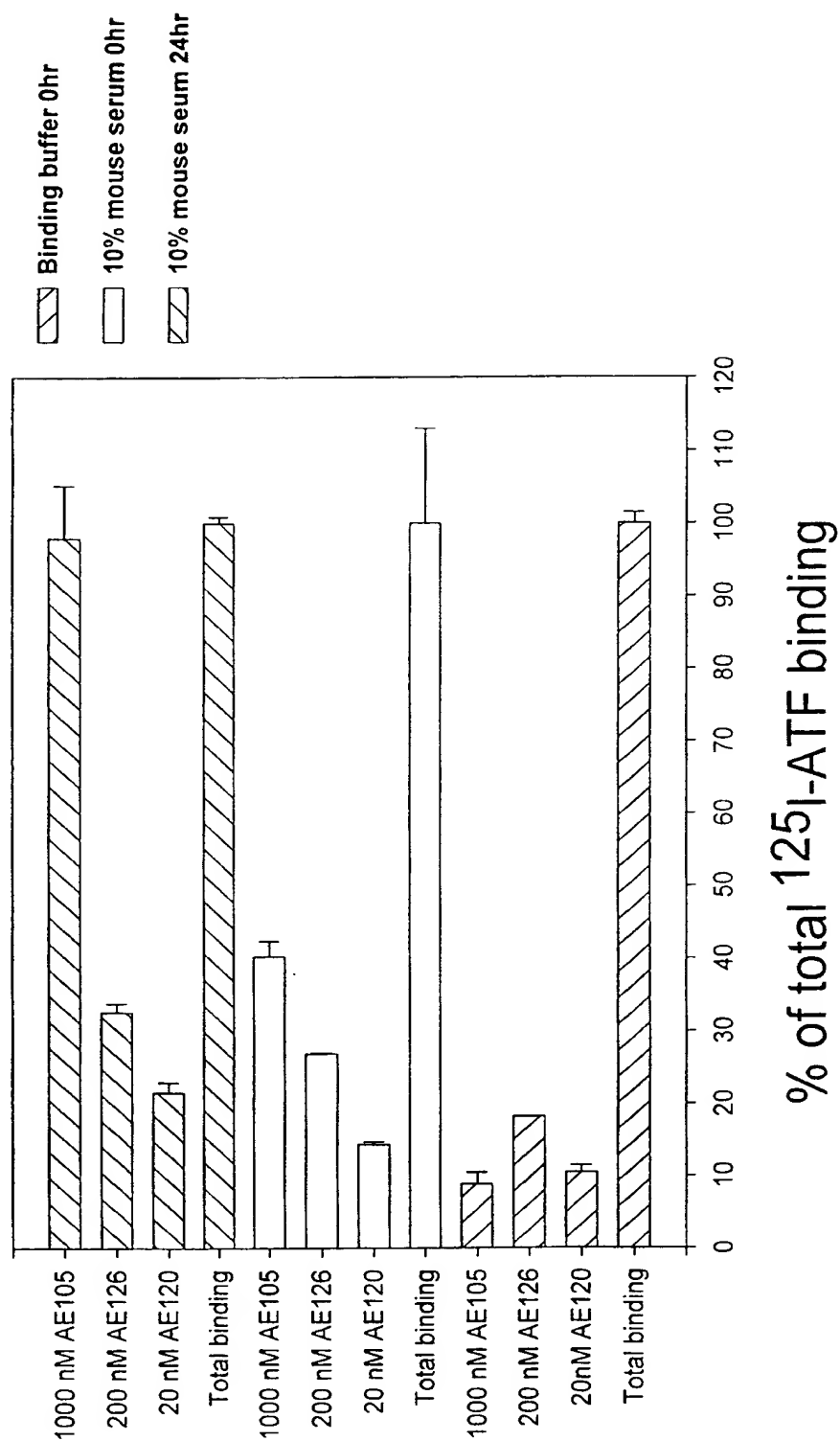


Figure 6

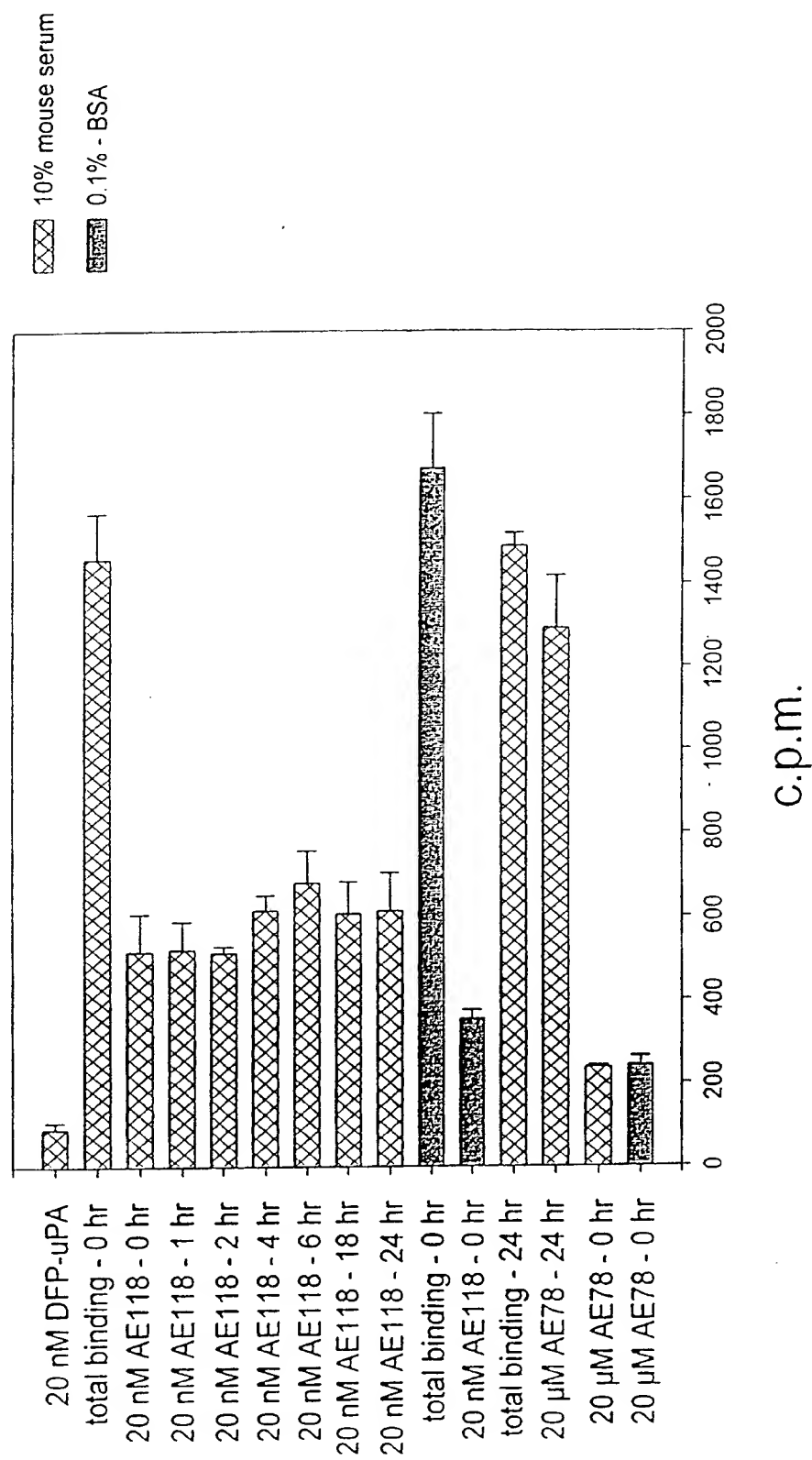


Figure 7

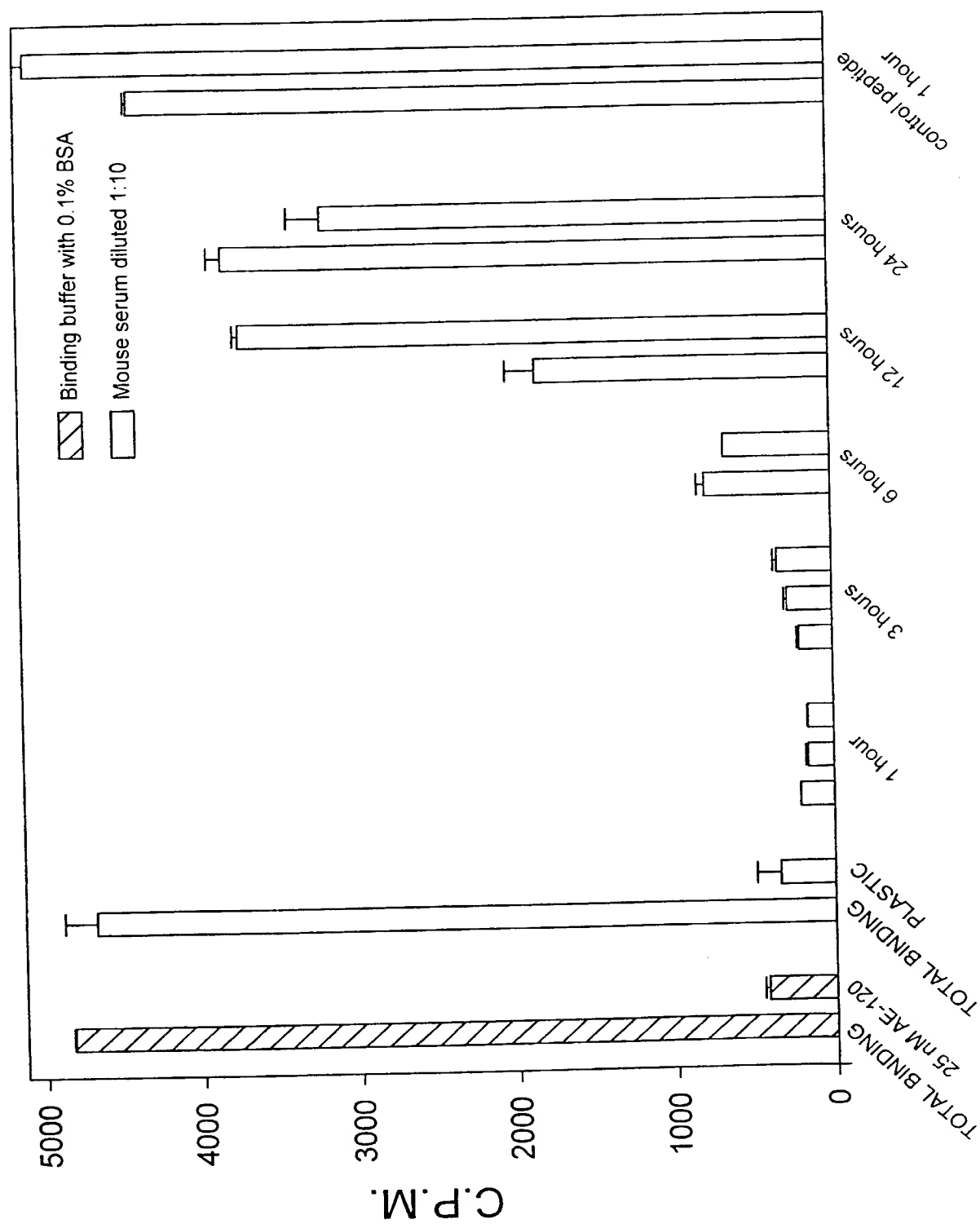


Figure 8

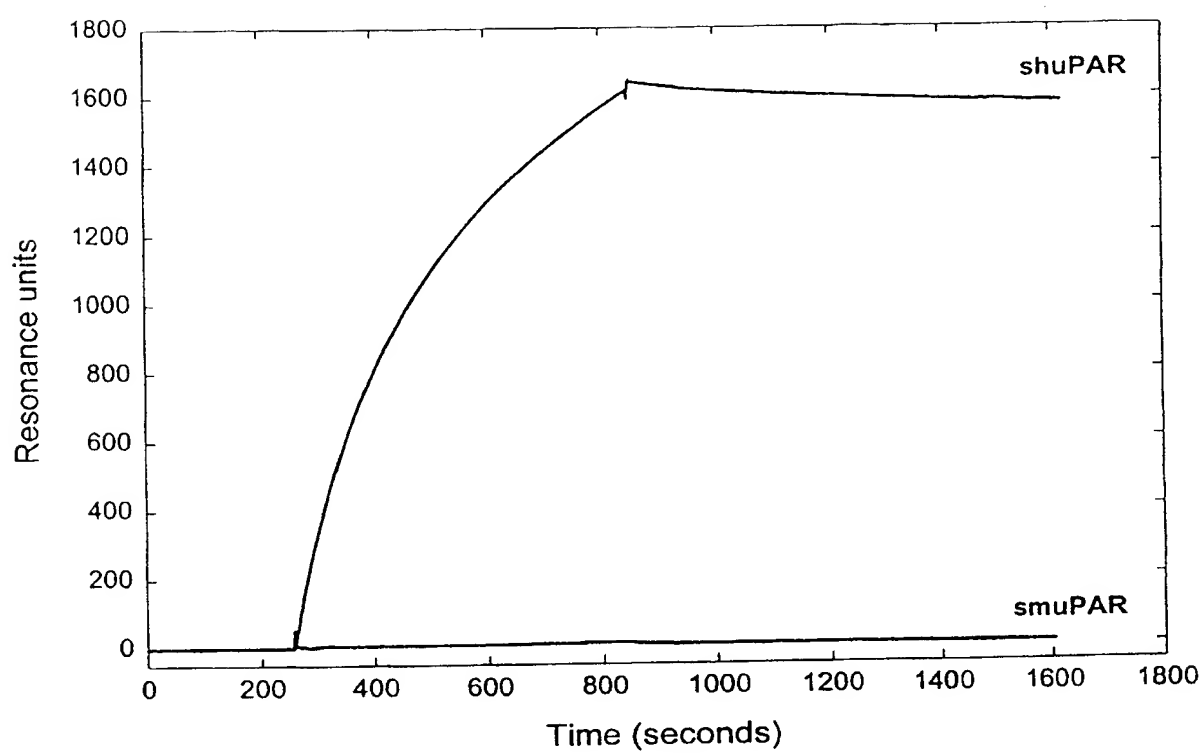


Figure 9

huPA/antagonist	★ ★ ★ ★ ★	★	↓	% identity
Human (+/+)	WHEKTNRTLSYRTLKITSLTEV	★	QHHLDFSMNHIDVS	(100)
Hamster (+/-)	WHEKTNRTMSYRVSKII SLAEV		QGHVDFLLSHPNIS	(63.5)
Mouse (-/-)	WHEKTNRTMSYRSMII SLTEV		QGHVDFPTHNVSVS	(62.2)
Rat (-/?)	WHEKTNRTMSYRMSVIVSLTEV		QGHVDFQTHNLSTS	(62.5)
Bovine (?/?)	WHDKTNRSM SYRADQIITLSEV		QSHVEFDLTHVNVS	(61.6)
		uPAR domain I loop 3	uPAR domain III loop 3	

Figure 10

